Protoplast fusion for intraspecific transfer of cytoplasmic male sterility in perennial ryegrass

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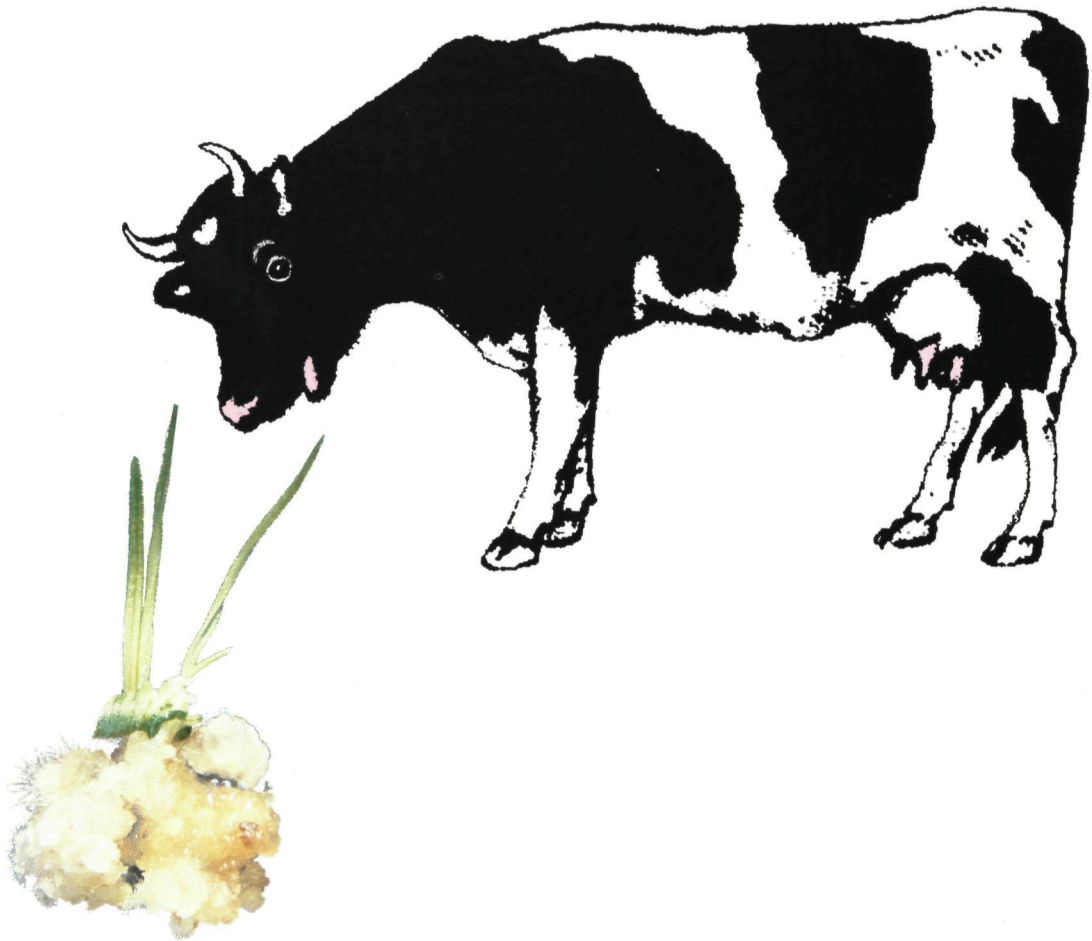

PROTOPLAST FUSION FOR INTRASPECIFIC TRANSFER OF CYTOPLASMIC MALE STERILITY IN PERENNIAL RYEGRASS

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PROTOPLAST FUSION FOR INTRASPECIFIC TRANSFER OF CYTOPLASMIC MALE STERILITY IN PERENNIAL RYEGRASS

Een wetenschappelijke proeve op het gebied van de Natuurwetenschappen, in het bijzonder de biologie

Proefschrift

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door

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CONTENTS

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GENERAL INTRODUCTION

Perennial ryegrass (*Lolium perenne* L.) is the most important forage grass in regions with a temperate climate. The existence of a wide range of variation in wild populations has allowed genetic improvement via conventional breeding (Wilkins, 1991). Furthermore, it is expected that crosses between selected inbred lines will produce F_1 hybrid seeds with improved agronomic characters, as a consequence of the occurrence of heterosis. Male sterility in one of the parents is an essential component of such crosses, because self-fertility of the female parent could restrict the necessary cross-fertilization. Since hand emasculation in grasses is very time consuming, male sterility in one of the parental lines is indispensable for the production of F_1 hybrid seed. Male sterility is a trait that causes a plant to produce unfunctional pollen. In perennial ryegrass a stable type of male sterility is available. This property originated from a cross between an F_4 hybrid of *L. perenne* X *L. multiflorum* and an autotetraploid *Festuca pratensis* (de Wit, 1973). From that cross two tetraploid male sterile plants were obtained. These plants were open-pollinated with predominantly diploid ryegrass and, after successive generations, male sterile plants still occurred in the off-spring populations. From that study it was concluded that a sterility-inducing cytoplasm and recessive nuclear restorer genes are needed for the expression of this type of male sterility, and is consequently considered as a type of cytoplasmic male sterility (CMS). According to the study of de Wit (1973) the sterilizing ryegrass cytoplasm originated from a plant of *Lolium perenne* collected in an old Dutch pasture.

CMS can be transferred to a selected breeding line by sexual crossing followed by repeated backcrossings. By this method, it takes ca. 6 years to obtain male sterile plants that are nearly identical to the original fertile (female) line. It is now generally accepted that cytoplasmic male sterility is encoded by the mitochondrial genome. This implies that transfer and substitution of mitochondria alone, from a sterile to a fertile line, should induce the expression of male sterility in the latter (Kumar and Cocking, 1987, Lonsdale, 1987, Rose et al., 1990). By asymmetric protoplast fusion techniques, the cytoplasms of two cells can be combined with the nucleus of only one of the parental lines and, after the sorting out of organelles and regeneration, plants with the desired nucleus/cytoplasm combination can be identified. Using this method, no hybridization of the nuclear genomes should take place and, as a consequence, the time-consuming cycles of back crossing may be avoided.

The aim of this research was the intraspecific transfer of CMS from the available male sterile perennial ryegrass line to selected, fertile breeding lines via protoplast fusion. The applicability of protoplast fusion techniques in plant

breeding programs can only be successful when protoplasts and fusion products of protoplasts can be regenerated to plants again. In cereals and grasses plant regeneration from protoplasts has only been obtained when fast-growing, regeneration-competent suspension cultures were used for the isolation of protoplasts. During the first part of this research program a procedure for the isolation, culture and regeneration of *Lolium* protoplasts was determined. During the second part, a method for asymmetric protoplast fusion was developed. In the first chapter an introduction and a review are presented of the achievements in tissue culture and genetic engineering in *Lolium* species as reported in the literature and with reference to the present study. In the subsequent four chapters a description and a discussion of the results of the present study on tissue culture of perennial ryegrass is presented. The last chapter deals with protoplast fusion and characterization of fusion products.

REFERENCES

Kumar A, Cocking EC (1987) Protoplast fusion: A novel approach to organelle genetics in higher plants. Amer J Bot 74(8):1289-1303

Lonsdale DM (1987) Cytoplasmic male sterility: a molecular perspective. Plant Physiol Biochem 25(3):265-271

Rose RJ, Thomas MR, Fitter JT (1990) The transfer of cytoplasmic and nuclear genomes by somatic hybridisation. Aust J Plant Physiol 17:303-321

Wilkins PW (1991) Breeding perennial ryegrass for agriculture. Euphytica 52:201-214

de Wit (1973) Cytoplasmic male sterility in ryegrasses (*Lolium* spp.) detected after intergeneric hybridization. Euphytica 23:31-38

CHAPTER 1

IN VITRO CULTURE AND MICROPROPAGATION OF RYEGRASSSES (*LOLIUM* SPECIES)

With: L.J. Beerepoot

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1. INTRODUCTION

1.1 Ryegrasses and their importance

The ryegrasses (*Lolium* spp.) can be classified into 8 different species. However, since these species are partly or fully interfertile, this classification is not always unequivocal (Breese and Tyler, 1986). Two of those species, perennial ryegrass (*Lolium perenne* L.) and Italian ryegrass (*Lolium multiflorum* Lam.) are very important in agriculture. Strictly annual forms of Italian ryegrass are often referred to as Westerwolds ryegrass (*Lolium multiflorum* Lam. ssp *westerwoldicum*). Hybrid ryegrass (*Lolium* x *hybridum* Hausskn.) is derived from artificial or naturally occurring crosses between Italian and perennial ryegrass. Perennial and Italian ryegrasses are diploid (2n=14), outbreeding and self-incompatible species. No naturally occurring tetraploids have ever been found. However, by means of colchicine treatment tetraploid varieties (4n=28) have been made artificially (Wit, 1959).

Grass is a very important world crop. Jones and Lazenby (1988) point out that in some countries, for example Great Britain, Australia and New Zealand, products from animals kept on grassland make a greater contribution to the value of agricultural production than does any cultivated crop. In temperate areas, perennial ryegrass and Italian ryegrass, together with their synthetic hybrid make up the major part of the newly sown grasses. Breese and Tyler (1986) state that they account for more than 85% of seed sales of agricultural grasses in Great Britain. According to estimates for the grass seed use in the EC in 1987-1988, 73% of the total used (100,000 tonnes seed per year) is ryegrass seed. This includes amenity grasses. Based on information from various industry experts, the US grass seed market in 1989 was estimated to be 240,000 tonnes, including amenity grasses. Of these, about 46% are ryegrasses. However, grass seed use, although very important for motivating trade and research, does not give a complete view on the importance of ryegrasses and the area under cultivation, since a major part of grasslands are only resown once every 4-10 years. Even pastures which are never resown can contain up to 100% ryegrass under favourable climatic conditions and with proper management.

1.2 Distribution and use of ryegrasses

Perennial ryegrass and Italian ryegrass are widely used as forage species in countries with temperate climates and sufficient rainfall in the growing season, e.g. Western Europe, New Zealand, South Africa and parts of Australia. In the USA, it is grown west of the Sierra Nevada and the Cascade Range, in the southern humid areas and northwards along the

Atlantic coast (Frakes, 1973). In Canada some ryegrass is used in the Atlantic Provinces and the south coastal part of British Columbia.

Ryegrasses are well suited for intensive farming practices and respond well to high fertilizer applications. Compared to other forage grasses, they produce forage with a high palatability and digestibility. Because of their quick establishment, they are more suitable then other grasses for overseeding and sod-seeding in an existing sward. Ryegrasses are also used as a green manure crop and for soil conservation purposes.

In those areas where severe frost, summer drought or heat prevents the survival of perennial ryegrass, Italian or Westerwolds ryegrass can be used as an annual forage crop or as a catch crop. For this reason, the use of Italian ryegrass is geographically more widespread than the use of perennial ryegrass.

Perennial ryegrass is used pure, or with other species in seed mixtures for permanent grassland intended for pasture, silage or hay. It can withstand heavy grazing quite well. The persistence varies with the climate, farming practices and the occurrence of pests. In favourable areas it seems to persist indefinitely, in other areas it is said to be only "quite satisfactory for three or four years" (Frakes, 1973).

Perennial ryegrass is used all over the world as a valuable species for home lawns, sports fields and recreational grounds. Its main advantages over other amenity grass species are the excellent wear tolerance and the fast germination and establishment. Disadvantages are the limited drought and heat tolerance and its demand for frequent cutting and fertilization. Modern cultivars of amenity ryegrass are finer leaved and grow slower than forage ryegrasses.

Italian ryegrass is used to give a high production of good quality silage or hay for one or two years. Persistent varieties of Italian ryegrass and hybrid ryegrass can last up to three years under favourable conditions. Westerwolds ryegrass is a short-living type, it will persist from only one cut up to a growing season.

1.3 **Diseases and pests**

Not much effort is spent on the chemical control of pests and diseases in grasses compared to many other crops. This type of control is often not paying and toxic residues can cause problems for grazing animals. Therefore, resistance breeding is the obvious solution to most disease problems. The outbreeding grasses, especially when they are sown in mixtures of species and varieties, are less vulnerable to epidemic diseases than genotypically uniform crops like cereals. Frequent cutting or grazing can stop spreading of diseases because infected plant parts are removed and less favourable conditions for the infection are left (Plumb, 1988).

The most important disease in ryegrass in most areas, crown rust (*Puccinia coronata*), is less damaging under intensive management with frequent cuts than under conditions giving a relatively slow growth rate. Crown rust occurs mainly in late summer and autumn and is promoted by high temperatures and high air humidity. A minor attack reduces the palatability of ryegrass, heavier attacks cause important yield losses and even plant death. Breeding for resistance to this disease is quite well possible, since considerable genetic variation is available within ryegrass and selection for resistance is relatively easy, both in the field and in greenhouse tests.

Winter damage in perennial or biennial ryegrass can be caused by frost but also by different snow moulds (*Gerlachia nivalis* and *Typhula* spp.), especially in areas with prolonged snow cover in winter. There are differences in susceptibility between varieties. A selection method for increasing resistance to snow mould has been described by Jonsson and Nilsson (1986).

In a perennial grass sward, individual tillers do not live for years. New tillers grow from axillary buds on older tillers. Because of this continuous vegetative propagation, virus infection is quite common, especially in old grass swards. Of the many different viruses affecting ryegrasses, barley yellow dwarf virus and ryegrass mosaic virus are the most important. They cause great damage and sometimes death to individual plants. In a sward, however, effects are less catastrophic because uninfected neighbours will fill the gaps left by slower growing virus-infected plants. Differences in susceptibility between varieties do exist, however, breeding for virus resistance or tolerance is difficult. Virus resistance might be one of the first important goals for genetic engineering.

Insects can be especially harmful during the establisment of newly sown grass into old grassland after ploughing or sod-seeding. Frit fly larvae (*Oscinella frit*) bore into grass tillers and feed especially on the young tissue around the meristems. Leatherjackets, larvae of craneflies (*Tipula* spp.) are common in northern Europe. They feed on underground parts of ryegrass plants and on seedlings. Resistance to those insects is unknown in ryegrasses. Genetic engineering might offer possibilities for the introduction of insect resistance. Only some of the most common pests and diseases are mentioned here. More comprehensive information is given by Plumb (1988).

1.4 Breeding and propagation

Commercial ryegrass varieties are panmictic populations of heterozygous plants, expressing a certain level of uniformity for morphological characteristics such as date of ear emergence, length of culms and growth habit. Most modern varieties are based on a limited number of selected plants or clones. They are often referred to as synthetic varieties.

Seed production after random mating between the originally selected clones is often called syn-1. This seed can be sown to raise the next seed generation syn-2, etc. Commercial seed is usually syn-5 or syn-6 seed. During successive generations, varieties should be stable in performance and morphological characteristics. However, this is not always the case (Hayward and Abdullah, 1985).

Selection of parent plants for new varieties usually starts in heterogeneous populations. These populations can be F_2 or F_3 generations derived from crosses, ecotypes collected from very old pastures, or existing varieties. Wild ecotypes provide a seemingly limitless gene-pool of variation on which the breeder can draw (Tyler,1987).

Table 1. One of the breeding methods used by Barenbrug Holland BV for forage breeding ryegrass, based on mass selection

Year	
1	Cross between two varieties
2	Production of F_2 seed
3	$\pm$ 400 F_2 plants per cross are vegetatively multiplied to clones. Clones are planted in a selection trial with a between-plant distance of 12.5 cm. Cutting and fertilizing are according to farming practice
4,5	Observation of persistence, winter hardiness, disease resistance, and early spring growth
6	Transplanting of 10% superior clones to disease nursery. About half of them are rejected, mainly on crown rust susceptibility. In autumn, the remaining 5% are planted in an observation trial, ten plants per clone, distance between clones 50 cm
7	Observation of morphological characteristics such as heading date. Formation of groups of two to six similar clones. Transplanting to irrigated plots in rye just before flowering. Syn1 seed production in isolation
8	Sowing of yield trials at several locations
9,10	Yield assessment and observation of trials. Candidate varieties looking better than control varieties are sown for seed multiplication in the autumn of year ten
11	Harvest of syn2 seed, which is used to start National List tests

Selection methods can be based on mass selection between plants or clones (Table 1). Mass selection is a cheap and easy method, enabling a breeder to cope with large numbers of genotypes. A disadvantage is that performance in spaced-plant conditions, even with narrow spacing, can be quite different from performance in a sown sward. Also, it is not easy to

estimate yield differences between plants or clones. Because of the heterozygous nature of the tested plants, phenotypically good plants can reveal bad characteristics in the next generation. Nevertheless, mass selection for phenotypic performance has proven to be successful in developing varieties in ryegrass at our breeding station.

Several selection methods have been published for use in ryegrasses involving tests of sown progeny of plants instead of testing the plants themselves. Raising enough seed for use in replicated field trials is possible using a topcross or a polycross. Utz and Oetler (1978) used a topcross in which an excess pollen cloud from a sown variety pollinates the genotypes to be tested. Frandsen and Frandsen (1948) and Schaepman (1952) used a polycross method in which progeny seed is harvested from clones, each plant of which is positioned randomly in such a way that every genotype is pollinated by most of the other genotypes of the polycross group.

Both the topcross and the polycross require that mother clones can be kept alive until the progeny tests have been finished and evaluated. Because of severe frost in some years, virus diseases, or lack of persistence, this can be very difficult in trial fields. Meristem culture as described in 2.5 is very useful for this purpose.

One of the ways to increase the selection response in breeding programs is by maximizing the additive genetic variation between selection units (Hallauer and Miranda,1981). Selection between homozygous plants would be much more effective than between heterozygous ones. However, inbreeding in the self-incompatible ryegrasses is difficult. Utz and Oetler (1978) only managed to get 284 S6-lines by single seed descent starting with 2600 initial selfings.

1.5 **The application of biotechnology in ryegrass breeding**

Plant biotechnology covers the area of research that combines tissue culture and molecular techniques with the aim of obtaining plants with improved agronomical traits. In this respect the availability of these techniques should be considered as an additional tool in current breeding programs. The possible benefits of biotechnology for grass breeding will be discussed below, a detailed description of the methods and the achievements in *Lolium* will be given in Chapter 2 of this review.

Micropropagation. Plants can be regenerated "in vitro" from cultured meristems. In ryegrass breeding this technique is used to preserve plant genotypes and to eliminate viruses.

Somatic hybridization and cybridization. Combining techniques for protoplast culture and regeneration with methods for protoplast fusion offers additional possibilities for plant breeding. Protoplast fusion can be accomplished between protoplasts from one species (intraspecific), between

protoplasts from related species (interspecific) or between distantly related species (intergeneric). After fusion the fused protoplasts (hybrid cells) need to be regenerated to mature (hybrid) plants. Initially, it was expected that this technique could be used to overcome sexual crossing barriers to produce novel species. However, it was shown that although hybrid plants could be obtained between e.g. crop plants and distantly related wild species, the hybrids often express undesirable traits like low seed fertility (Glimelius et al., 1991). A more promising way to exploit hybrid plants from such fusions is to use them as bridges to transfer valuable traits (e.g. disease resistance) from the alien species to the crop plant.

By asymmetric fusion hybrids are obtained which contain the cytoplasm genomes (chloroplasts, mitochondria) of both parental lines and the nuclear genome of only one of the two lines. To discriminate, such hybrids are referred to as cybrids. Asymmetric cybridization involves fragmentation or elimination of the nuclear DNA of one of the parental lines prior to fusion. This can be accomplished by irradiation (gamma, X-ray) or enucleation by centrifugation respectively. The mixed cytoplasms in the cybrid cells will segregate during subsequent mitotic divisions, allowing the proliferation of cells with novel combinations of nuclear and cytoplasm genomes. This direct method for cytoplasm transfer has proven valuable for the transfer of mitochondrion- and chloroplast-encoded traits, e.g. within *Brassica* species (Glimelius et al., 1991), circumventing the need of repeated back crosses.

For the commercial production of hybrid seeds, male sterility in one of the parental lines is indispensible. In perennial ryegrass a type of male sterility occurs, which can be stably maintained and is maternally inherited (cytoplasmic male sterility, CMS). This sterile line could be used as cytoplasm donor in asymmetric fusion experiments to transfer male sterility to other perennial ryegrass breeding lines.

Haploids. Haploid plants can be regenerated from cultured anthers, microspores and ovaries. The production of homozygous lines by anther culture could enhance selection efficiency in perennial ryegrass (see section 1.4). If hybrid varieties of perennial ryegrass become available (Utz and Oettler, 1978), anther culture will be useful for producing inbred lines. In breeding programs forced self-fertilization is used to make inbred lines. However, this method selects strongly against the naturally occurring self-incompatibility in *Lolium*. When such inbred lines are polycrossed or topcrossed, a certain unpredictable proportion of the progeny seed can come from self-fertilization, leading to wrong estimations of breeding values (Utz and Oettler, 1978). Producing homozygous plants by anther culture could possibly overcome this problem, since no selection against self-incompatibility takes place.

Transformation. Successful transformation is achieved when genes of interest are transferred, stably integrated and

expressed in the transformed plant and its off-spring (Potrykus, 1990). A limited number of agronomically important genes is yet available and has been transmitted successfully to an even more limited number of mainly dicotyledoneous crop species (Vasil,1990B). These genes confer resistance to several herbicides, insects and viruses, and as discussed in section 1.3 transformation of ryegrass with these traits could lead to improved varieties.

2. IN VITRO APPROACHES

2.1 Introduction to tissue culture studies in *Lolium*

As mentioned in section 1.5 the application of tissue culture and molecular techniques in plant breeding programmes of *Lolium* offers promising perspectives.

A well-established method for clonal propagation and long-term storage of genotypes by meristem tip culture has been available for more than 10 years. Recently, research has concentrated on the culture of anthers and haploid plants have been obtained from several *L. perenne* varieties. Throughout the last 15 years, results have been reported, which demonstrate that callus with a capacity to regenerate plants can be initiated readily from all (somatic) explants of *Lolium* that have been tested.

However, several techniques of genetic manipulation, e.g. somatic hybridization and direct gene transfer to protoplasts, require the availability of a procedure for protoplast isolation, culture and plant regeneration (Vasil,1988; Bajaj 1989). The general approach in gramineous species has been to determine the optimal conditions for callus induction and plant regeneration from explants. Morphogenic callus is then used for the initiation of suspension cultures, which are, once established, a suitable starting material for the isolation of protoplasts. Throughout the different "in vitro" stages, culture conditions must be determined which permit the tissues to retain and express a morphogenic capacity. In *Lolium* successful establishment of morphogenic cell suspension cultures has been reported and plants have also been regenerated from cell suspension-derived protoplasts of *L. perenne* and *L. multiflorum*.

The following chapters describe the culture conditions, techniques, culture media and results of recent advances concerning tissue culture studies and their applications in *Lolium*. The results are discussed with reference to the progress made with other gramineous species.

Plant species	Inoculum	Medium[a] (mg/l)
Lolium multiflorum	meristem tip	MS + kin (0 2)
L. multiflorum	meristem tip	MS + 2,4-D (0 1) + kin (0 2)
L. perenne	meristem tip	MS + 2,4-D (0 1) + kin (0 2)
L. multiflorum	meristem tip	MS + 2,4-D (2) + BAP (0 2) + CH (100)
L. multiflorum	root	LS + 2,4-D (5)
L. multiflorum	root	MS + 2,4-D (2) + BAP (0 2) + CH (100)
L. temulentum	root	LS + 2,4-D (5)
L perenne	root	LS + 2,4-D (5)
L. rigidum	immature embryo	MS + 2,4-D (10) + CH (0 1%)
L. perenne	mature embryo	MS + 2,4-D or Dicamba + BAP (0,5)
L. multiflorum	immature embryo	MS + 2,4-D (2) + BAP (0 2)
L perenne + L. multiflorum	immature seed	NO + 2,4-D (1 5) + IAA (6 5) + zeatin (0 25)
L perenne	mature seed	MS + 2,4-D (10)
L perenne	mature seed	MS + 2,4-D (5)
L multiflorum	immature inflorescence	MS + 2,4-D (2)
L multiflorum	immature inflorescence	MS + 2,4-D (2) + BAP (0 2) + CH (100)
L perenne	immature inflorescence	MS + 2,4-D (2) + BAP (0 2) + CH (100)
L. multiflorum	immature inflorescence	MS + 2,4-D (5)
L. perenne	immature inflorescence	MS + 2,4-D (5)
L. multiflorum	node	MS + 2,4-D (2)
L multiflorum x Festuca arundinacea hybrids	internode	MS + 2,4-D (2/4)
L. multiflorum	leaf	MS + 2,4-D (2/4)
L perenne x L. multiflorum	leaf	MS + 2,4-D (2) + BAP (0 2) + CH (100)
L multiflorum	suspension culture	mod MS + IAA (6 5) + 2,4-D (1 5) + kin (2 15)
L. multiflorum	suspension culture	MS + 2,4-D (2)
L. multiflorum	suspension culture	MS + 2,4-D (5/6)
L multiflorum	suspension culture	MS + 2,4 D (5→2)
L perenne	suspension culture	MS + 2,4-D (5/6)
L multiflorum	protoplasts	MS + 2,-D (0 0 25)
L multiflorum	protoplasts	mod MS + 2,4 D (0 1) + BAP (0 1) + glucose + mannitol
L perenne	protoplasts	mod MS + 2,4 D (0 1) + BAP (0 1) + glucose + mannitol
L. perenne	protoplasts	RY-2[b] + 2,4 D (4 4) + glucose + FC (0 8%) + CM (50%)
L multiflorum	anther	complex medium[b]
L. perenne	anther	complex medium[b]
L. temulentum	anther	complex medium[b]

[a] The simple medium for initial culture is given Different media have been used for maintenance and plant regeneration Numbers in parenthesis are mg/l

[b] The media used are composed of parts of formerly described (MS, LS) media with many additives For detailed description see references

[c] For detailed references see section References

Growth response	Reference[c]
Plants	Dale (1975, 1980d)
Plants	Dale (1977ab)
Plants	Dale (1977a)
Callus and plants	Jackson and Dale (1988)
Callus and roots	Atkın and Barton (1973)
Callus and plants	Jackson et al. (1986, 1988)
Callus	Atkın and Barton (1973)
Callus	Atkın and Barton (1973)
Callus and plants	Skene and Barlass (1983)
Callus, embryos and plants	Dale (1980b)
Callus and plants	Schmidt and Posselt (1990), Schmidt (1991)
Callus and plants	Ahloowalia (1975)
Callus and roots	Torello et al. (1983)
Callus and plants	Torello and Symington (1984)
Callus, embryos and plants	Dale et al (1981)
Callus and plants	Dale and Dalton (1983)
Callus and plant	Dale and Dalton (1983)
Callus, embryos and plants	Creemers-Molenaar et al. (1988b)
Callus, embryos and plants	Creemers-Molenaar et al (1988b)
Callus, embryos and plants	Dale et al (1981)
Callus and plants	Kasperbauer et al. (1979)
Callus and plants	Kasperbauer et al (1979)
Callus and plants	Jackson and Dale (1988)
Callus and embryos	Ahloowalia (1975)
Callus and plants	Jones and Dale (1982)
Callus, embryos and plants	Dalton (1988b), Creemers-Molenaar et al. (1989)
Callus, embryos and plants	Rajoelina et al. (1990)
Callus, embryos and plants	Dalton (1988b), Creemers-Molenaar et al. (1989), Zaghmout and Torello (1990b)
Callus	Jones and Dale (1982)
Callus and plants	Dalton (1988ab)
Callus and green plantlets	Dalton (1988ab)
Callus and plants	Creemers-Molenaar et al (1989)
Callus and plants	Niizeki and Oono (1977), Nitzche and Wenzel (1977), Pagniez and Demarly (1979), Bante et al. (1990, 1991)
Callus and plants	Stanis and Butenko (1984), Olesen et al. (1988), Bante et al. (1990, 1991), Boppenmeyer et al. (1989), Halberg et al. (1990), Hayward et al. (1990)
Callus and plantlets	Rose et al. (1987)

2.2 **Sterilization of the explants**

2.2.1 *Seeds*

If young leaves or roots are to be used as the explant material, plantlets can best be grown under aseptic conditions. The seeds are surface sterilized with a 50% (v/v) commercial sodium hypochlorite solution 20 min. After rinsing several times in sterile tap water the seeds are germinated under sterile conditions. For callus initiation from seeds or embryos the same sterilization procedure should be followed. Heavily contaminated seeds can be treated two times with a 100% (v/v) sodium hypochlorite solution, interrupted by soaking in sterile water for 2-7 days (Dalton,1988A). Dehusking of the seeds in 50% (v/v) sulphuric acid prior to sterilization has been shown to enhance callus initiation from mature seeds in *L. perenne* (Torello et al, 1983).

2.2.2 *Plants*

Tillers from field- or greenhouse-grown plants are surface sterilized by dipping the tillers into 70% (v/v) ethanol for 2-10 s, and then into a 50% (v/v) sodium hypochlorite solution containing 0.01% (v/v) Tween 80 for 20 min. After rinsing 5-6 times in sterile tap water the immature inflorescences or meristem tips are excised aseptically. For the culture of anthers, spikes are sterilized as described for immature inflorescences or with 0.1% (w/v) mercuric chloride for 8-20 min, followed by three washes in sterile water.

2.3 **Composition of culture media**

2.3.1 *Basic constituents*

Table 2 shows that MS medium (Murashige and Skoog, 1962) is the basal medium that has been used most often for micropropagation (meristem tips) and callus induction from explants of *Lolium*. For callus induction, the MS medium is usually supplemented with 0.8% agar, 3% sucrose, varying concentrations of growth regulators (2,4-D, IAA, kinetin, zeatin and/or BAP) and complex nutrients (coconut milk and casein hydrolysate). For the direct regeneration of plantlets from meristem tips a simple MS-based medium supplemented with 0.2 mg/l kinetin can be used. The pH of the medium is adjusted to 5.8 by the addition of 0.1 M HCl or 0.1 M NaOH. The complete medium can be sterilized by autoclaving for 15 min at 121^{o}C, but to prevent pH and other medium changes it is recommended to filter-sterilize the nutrients as a concentrated solution, which can be added to the autoclaved agar solution at a suitable temperature.

2.3.2 *Growth regulators*

In tissue culture studies on cereals and grasses auxins have proven to be indispensable for triggering callus initiation and to maintain established callus cultures. In

Lolium, the synthetic auxin 2,4-D (2,4-dichlorophenoxyacetic acid) is effective for callus initiation and callus maintenance. The optimal concentration depends on the explant type and ranges from 2-10 mg/l. The effect of the 2,4-D concentration on callus induction and regeneration has been determined for immature inflorescence cultures in *L. perenne* and *L. multiflorum* (Creemers-Molenaar et al,1988B). It was concluded that for the concentration range tested (2.5-15 mg/l) no optimal concentration could be determined for callus induction. However, in *L. perenne* higher concentrations of 2,4-D during the callus initiation period resulted in an increased ratio of regenerated albino shoots versus regenerated green shoots. Similar results were obtained with another synthetic auxin dicamba (3,6-dichloro-2-methoxy benzoic acid) in suspension-derived callus of rice (Zimny and Lörz,1986).

Several studies compared the effect of different auxins on callus induction, maintenenance and plant regeneration from explants in cereals and grasses. In sugarcane the synthetic auxin picloram (4-amino-3,5,6-trichloropicolinic acid) was superior to 2,4-D for the induction of morphogenic callus and the maintenance of long-term regeneration-competent callus (Fitch and Moore, 1990). In rice the phenoxyacetic acid-derivative 4FPA (4-fluorophenoxyacetic acid) was superior to 2,4-D with respect to callus induction and long-term maintenance of the regeneration competence (Yasuda et al., 1990).

Only few studies compared the effect of different auxins on callus induction from explants in *Lolium*. Schmidt (1991) showed that the proportion of embryo-derived calli that formed regenerants increased when dicamba was used instead of 2,4-D in the callus induction medium. However, the effect of different auxins was dependent on the genotype. For callus induction from leaf-tip and embryo explants from *L. multiflorum* dicamba was more effective than 2,4-D (Conger et al,1982). NAA and picloram were also more effective, but these auxins had to be applied at higher concentrations and the calli showed a high tendency for root formation. In the sole presence of the natural auxin IAA, no callus was formed on leaf-tip or embryo explants of *L. multiflorum*. Callus induction frequency and callus growth from embryos of *L. multiflorum* on media with 2,4-D or another synthetic auxin 2,4,5-T were comparable, but again more roots were formed in the presence of 2,4,5-T (Conger et al,1978).

However, once a suitable explant is chosen, it is not callus induction but the loss of regeneration competence in long-term callus and suspension cultures which is a problem in *Lolium*. Considering the above-discussed positive effects of picloram and 4FPA in sugarcane and rice respectively, the application of these auxins in callus and suspension cultures in *Lolium* needs further research.

In addition to 2,4-D the callus induction medium for explants of *Lolium* has also been supplemented with low concentrations of cytokinins (Table 2). Schmidt and Posselt (1990) showed that the frequency of plant regeneration from mature embryos increased with the concentration of BAP (0-0.5 mg/L) in the callus induction medium. The number of regenerants per callus was not influenced by BAP. Furthermore, the effect of BAP was dependent on the composition of the basal MS medium (type of auxin and concentration of thiamine). The effects of cytokinins in callus cultures of other gramineous species are variable. In wheat callus cultures cytokinins inhibited growth (Dudits et al,1975). In protoplast-derived callus of *Panicum maximum* zeatin favoured the formation of organized embryoids (Lu et al, 1981). Low concentrations of kinetin and zeatin induced shoot formation from protoplast-derived callus in rice (Kyozuka et al.,1987).

2.4 **Physical culture conditions**

Most tissue culture studies in *Lolium* have been performed at a temperature of 25°C, however, with reference to the light regime no consistency in conditions is evident.

During the initial culture period callus cultures from explants of *Lolium* may be incubated either in the dark (Creemers-Molenaar et al.,1988B) or in diffuse light (Kasperbauer et al.,1979, Dale et al.,1981). Suspension cultures of *L. multiflorum* and *L. perenne* were kept in diffuse light continuously (Dalton,1988A, Creemers-Molenaar et al.,1989). Such light-grown suspension cultures often contained cell aggregates with green areas, but other characteristics, like plant regeneration and growth rate, were the same for dark and light grown cultures (Creemers-Molenaar, unpublished). For the regeneration of shoots, callus may be cultured in continuous light (Dale,1980B, Dale and Dalton,1983, Jackson et al.,1986, Creemers-Molenaar et al.,1988B, Rajoelina et al., 1990) or in a light/dark regime (Skene and Barlass,1983, Torello and Symington,1984).

As reported for rice (Abdullah et al.,1986) and sugarcane (Ho and Vasil,1983) exposure of morphogenic callus to the light at an early stage resulted in precocious germination of somatic embryos. The effects of different light regimes on tissue cultures of *Lolium* have not been reported, but obviously need further experimentation.

2.5 **Meristem culture**

For the preservation of grass genotypes, meristem culture can be used as has been published by Dale (1975,1977A,1980A). Meristem culture can also be used to eliminate ryegrass mosaic virus and other viruses in valuable ryegrass plants (Dale,1977B).

At our breeding station, meristem culture is applied to

plants, which have produced seed for progeny tests during the summer. In autumn, plants are put inside an unheated, but frost-free greenhouse. Insecticides are applied against frit fly larvae (Oscinella frit), because they feed on the inner parts of the tillers, where the meristems are situated. Two weeks before meristem culture work starts, the plants are transferred to a greenhouse at 20°C to stimulate growth and the development of shoot tips.

From about 10 healthy, vegetative tillers of every genotype the basal 8 cm is cut. Roots are cut back to ±1 cm. These tillers are surface sterilized as described in 2.2. Shoot tips are excised aseptically from the tillers under a stereo microscope. For virus elimination purposes, the dissected part should be as small as possible, containing only the meristem tip. For preservation purposes, larger parts of shoot tips, 1-2 mm long, may be excised (Fig. 1). Per genotype, 8 shoot tips are placed seperately in a glass tube containing about 12.5 ml solid culture medium as described in 2.3.1. Glass tubes containing meristems are placed in a growth room at 20°C with ± 2000 lux continuous light from fluorescent Philips TL 83 lamps.

Not all shoot tips develop into plants and some may still be infected. Therefore, 8 shoot tips per genotype are cultured to obtain at least 3 successful ones. Normally, within a few days the first leaf starts to develop. After about 8 weeks, most shoot tips have developed into complete shoots with roots. At this stage, 3 tubes with successfully established, uninfected shoots per genotype are put into coldstorage at 4°C under continuous light (± 1000 lux, Philips TL 57 RS). The shoots can be maintained in cold storage for 18 months before subculturing is needed.

During cold storage, the plants grow slowly and form several new tillers which enables some vegetative propagation. For this a 2 cm piece of the new tiller, containing the shoot tip, is placed with the lower half in fresh culture medium. Usually, growth starts immediately and after 4 weeks plants are returned to cold storage again.

Subculturing can be repeated indefinitely. At our breeding station, we have kept Italian ryegrass clones in good condition by meristem culture for 9 years, without any loss of vigour. This would have been very difficult, if not impossible, using trial fields or greenhouses.

Re-establishment of plants in soil is relatively easy. Glass tubes with desired genotypes are collected from cold storage and plants are taken out of their tubes. Culture medium is removed under running tap water. Plants are put in normal, unsterilized potting compost. They remain covered for about a week to retain a high air humidity. Several hundreds of selected clones so far have been re-established and so far no genotype has failed to establish itself in soil again.

Table 3. A time course of the procedure for callus initiation and plant regeneration from immature inflorescence explants of *L. multiflorum* and *L. perenne*.

week	
1	After sterilization of the tillers, the immature inflorescences are excised and 2mm long segments are cultured on solid MS medium, supplemented with 5 mg/l 2,4-D. The cultures are incubated in the dark at 25°C.
4	Explants which have formed watery callus are transferred to fresh medium .
6-8	At the end of the second culture period a number of explants have formed areas of white, compact callus. To enhance the proliferation of compact callus, this is excised and subcultured two times onto fresh medium, at two weekly intervals.
10	At the end of the fourth subculture period, most of the compact calli have become nodular, showing the onset of somatic embryogenesis. To stimulate further development of pro-embryogenic structures, compact and nodular calli are subcultured onto MS medium with 0.2 mg/l 2,4-D.
13-19	At this stage well-developed somatic embryos can be observed. Compact and embryogenic calli are subcultured onto MS medium without 2,4-D in the light.
16-19	During this period many shoots regenerate asynchronously from the callus. Green as well as albino shoots are observed. Shoots regenerate via the formation of somatic embryos or directly from compact callus. After 3-6 weeks green shoots are removed from the callus and transferred to culture vessels with the same medium. The cultures are kept at 20°C under continuous light from fluorescent Philips TL 83 lamps.
19-22	Rooted plantlets are transferred to pots with soil in the greenhouse, under conditions that prevent evaporation.
23	Plants are grown in the greenhouse or in the field and can be maintained as normal plants.

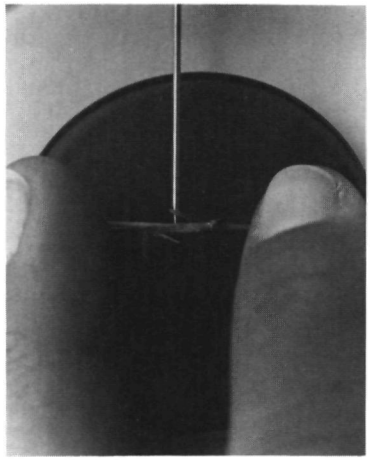

Fig. 1. A shoot tip, excised from a ryegrass tiller, to be used in meristem culture.

2.6 **Callus initiation and plant regeneration**

Cultured explants of *Lolium* initially form a watery type of callus, which is considered to be non-morphogenic. However, parts of these watery calli have been observed to form centers of compact morphogenic callus. Plant regeneration has been shown to originate mainly from this compact callus via the formation of somatic embryos. However, less frequently shoots have also been observed to regenerate directly from compact callus (organogenesis).

Table 2 shows that callus cultures have been established from almost all plant parts of *L. multiflorum* and *L. perenne*. A representative time course of the procedure for callus initiation and plant regeneration is given in Table 3. Depending on the explant type culture periods will differ from this example, but the developmental process is the same.

Vasil (1987) reviewed which factors primarily influence the callus response and regeneration from explants of gramineous species. It was concluded that the physiological and the developmental state of the explant at the time of exision control the morphogenic reponse to a larger extent than its genotype. In cultured tissues of gramineous species only the meristematic cells are able to proliferate and form regeneration competent cells. The presence of too many differentiated cells in the explant might suppress the morphogenic response of competent cells (Vasil, 1987).
The importance of the developmental stage and the type of explant was demonstrated in perennial and Italian ryegrass (Dale,1980B, Joarder et al, 1986, Creemers-Molenaar et al., 1988B). In *L. perenne* explants from immature inflorescences at

a defined developmental stage showed a high response for callus induction (80-100%) and compact callus formation (56-100%) (Creemers-Molenaar et al.,1988B). In comparison less than 10% of cultured mature embryos of *L. perenne* formed compact callus (Creemers-Molenaar, unpublished).

2.7 **Cell suspension cultures**

2.7.1 *Establishment of cell suspension cultures*

In gramineous species suspension cultures have proven to be the most suitable starting material for the isolation, culture and regeneration of protoplasts. Such suspension cultures should be finely dispersed, fast growing and morphogenic. Suspension cultures with the capacity to regenerate green plants have been obtained from several gramineous species, including: *Oryza sativa*, japonica genotypes (Abdullah et al.,1986, Toriyama et al.,1986, Yamada et al.,1986 and Kyozuka et al.,1987,1988) and indica genotypes (Lee,et al.,1989, Wang et al.,1989, Datta et al.,1990), *Zea mays* (Kamo and Hodges,1986, Vasil and Vasil,1986, Rhodes et al.,1988, Prioli and Sondahl,1989), *Saccharum officinarum* L. (Ho and Vasil,1983), *Triticum aestivum* L. (Ahuja et al.,1982, Harris et al.,1988, Vasil et al.,1990A), *Dactylis glomerata* (Gray et al.,1984), *Festuca arundinacea* Schreb. (Dalton,1988AB, Takamizo et al.,1990, Rajoelina et al.,1990), *Festuca rubra* L. (Zaghmout and Torello,1989), *Lolium perenne* (Dalton,1988AB, Creemers-Molenaar et al.,1989, Zaghmout and Torello,1990B) and *Lolium multiflorum* (Dalton,1988AB, Creemers-Molenaar et al.,1989).

In *Lolium*, suspension cultures are initiated using immature inflorescence-derived compact callus or directly from 30-40 seed-derived embryos in liquid MS medium, supplemented with 5-6 mg/l 2,4-D. The cultures are incubated on a rotary shaker (100-140 rpm, 15mm amplitude) in the dark or in continuous indirect light (200-400 lux) at 25°C. Established suspension cultures are obtained 10-20 weeks after initiation and mainly consist of small pieces of compact callus with densely cytoplasmic cells. Young suspension cultures (<20 wks) are often embryogenic with somatic proembryos attached to the compact cell aggregates or freely suspended in the medium. These suspension cultures are maintained by weekly subculture of small cell aggregates to fresh medium.

Within a given plant variety different cell suspension cultures, each initiated with a number of genotypes, exhibit much variation in growth characteristics and the amenability to become an established culture. The results reported by Creemers-Molenaar et al. (1989), indicate that immature inflorescence-derived callus is more suitable for suspension culture initiation than seed-derived embryos. Depending on the variety, in *L. perenne* the percentage of suspension cultures initiated from embryos and callus that became established was

0-38 % and 20-100 % respectively.

2.7.2 *Plant regeneration from cell suspension cultures*

Young suspension cultures of *L. perenne* and *L. multiflorum* (<20 wks after initiation) have a high potential for plant regeneration. After plating on solid MS medium without 2,4-D up to 200 green shoots / g fresh weight suspension cell aggregates have been obtained in *L. perenne* (Creemers-Molenaar et al.,1989). However, most suspension cultures lose the ability to regenerate green shoots within 20-30 weeks after initiation. Most suspension cultures also regenerate albino shoots, and the frequency of albino shoots that regenerate tends to increase with suspension culture age, preceding complete loss of the regeneration potential. Loss of the regeneration capacity in long-term suspension cultures has been observed in most cereals and grasses and has stimulated research to maintain or restore the regeneration potential:

1) In a two year old suspension culture of rice, a three month pretreatment with 1.5% NaCl resulted in the enrichement of embryogenic cells, thus restoring the high regeneration competence of the culture (Binh and Heszky, 1990). Preliminary results with suspension cultures of perennial ryegrass did not confirm these results (Creemers-Molenaar, unpublished). NaCl treatment resulted in either browning and death of the culture or the slow proliferation of compact friable callus. This callus showed no characteristics of plant regeneration upon plating on solid medium.

2) In wheat suspension cultures lowering of the 2,4-D concentration induced the formation of roots. Removal of the root-forming callus and culture of the remaining callus at reduced 2,4-D levels resulted in the establishment of a long-term (2.5 years) regeneration-competent suspension culture (Wang and Nguyen, 1990).

3) Vasil et al. (1990A) stated that in wheat only the use of carefully selected, aged embryogenic callus leads to the establishment of long-term (1 year) regeneration-competent suspension cultures.

4) Creemers-Molenaar and van Oort (1990) showed that fast growing suspension cultures of perennial ryegrass can be stored for at least 6 weeks at 4°C without loss of the regeneration potential. When the same cultures were kept under standard conditions the regeneration capacity decreased as usual. Thus cold-storage of perennial ryegrass suspension cultures permits prolonged use of the cultures and is time-saving with respect to subculture regimes.

5) In callus cultures of alfalfa the replacement of part or all of the sucrose by maltose increased the formation of embryos and the regeneration of green plants (Strickland et al., 1987). The culture of wheat anthers in liquid medium with maltose instead of sucrose resulted in increased callus induction from microspores and increased the regeneration of green shoots from these calli (Orshinsky et al., 1990). In

perennial ryegrass anther culture the replacement of sucrose by maltose improved the percentage of anthers that formed callus (Bante, 1990, 1991). Replacement of part or all of the sucrose by maltose in the suspension culture medium did not affect the regeneration potential of perennial ryegrass suspension cultures (Creemers-Molenaar and van Oort,1990).
6) In perennial ryegrass, the frequency of immature inflorescence-derived calli forming albino shoots increased with increasing concentrations of 2,4-D in the callus induction medium (Creemers-Molenaar et al., 1988B). Lowering of the 2,4-D concentration to 2.5 mg/l or partial replacement of 2,4-D by the weaker auxin NAA in the suspension culture medium of perennial ryegrass did not improve the competence for plant regeneration (Creemers-Molenaar, unpublished).
7) Promising results have been obtained by Shillito et al. (1989), who demonstrated that the morphogenic potential of a maize suspension culture was retained after cryopreservation. After cryopreservation the suspension cultures could be re-established, and protoplasts isolated from such suspensions were able to regenerate into plants again. Regarding the short regeneration period of perennial ryegrass suspension cultures, and the more or less fruitless efforts to prolong this period, cryopreservation might be an essential step to maintain regeneration-competent suspension cultures in perennial ryegrass.

In conclusion, there is as yet no general procedure available for the establishment of long-term regeneration-competent suspension cultures in cereals and grasses. methods, as reported in the literature, often are only applicable for certain species, or for certain genotypes. Consequently, optimal tissue culture conditions will have to be determined for every monocot species.

2.8 **Protoplast isolation, culture and regeneration**

2.8.1 *Isolation of protoplasts*

Lolium protoplasts are isolated from actively-growing suspension cultures by treating the cells for 4 hours with an enzyme mixture containing 1% (w/v) Cellulase Onozuka RS and 0.1% (w/v) Pectolyase Y-23 in CPW-13M medium (Frearson et al.,1973). Different combinations of the former with Meicelase, Macerozyme R10 or Driselase have also been used, leading to similar results with respect to the yield of protoplasts. For overnight incubation the enzymes were used at half strength. Pure protoplast suspensions are obtained by sieving and washing of the protoplast-enzyme mixture. Protoplast yield from *L. perenne* suspension cultures varies from 0.1-14 million per g fresh weight and is dependent on the growth characteristics of the suspension culture.

Table 4. Plant regeneration from protoplasts in gramineous species. Protoplasts were isolated from callus-derived suspension cultures. In this table the explants which were used for callus induction are given.

Plant species	Explant type	References
Agrostis alba	seed	Asano and Sugiura, 1990
Dactylis glom.	leaf	Horn et al., 1988A
Festuca arund.	m embryo	Dalton, 1988AB
Festuca arund.	i embryo, leaf	Takamizo et al., 1990
Festuca rubra	seed	Zaghmout and Torello, 1990A
L. perenne	i infl, m embryo	Creemers-Molenaar et al., 1989
L. multiflorum	m embryo	Dalton, 1988A
Oryza sativa^{j}	leaf	Abdullah et al., 1986
Oryza sativa^{j}	anther	Toriyama et al., 1986
Oryza sativaj	seed	Yamada et al., 1986
Oryza sativa^{j}	seed	Kyozuka et al., 1987
Oryza sativa^{i}	seed, i embryo	Lee et al., 1989
Oryza sativa^{i}	seed	Wang et al., 1989
Oryza sativa^{i}	microspores	Datta et al., 1990
Saccharum off.	leaf	Srinivasan and Vasil, 1986
Saccharum off.	leaf	Chen et al., 1988
Triticum aest.	i embryo	Vasil et al., 1990A
Zea mays	i embryo	Rhodes et al., 1988
Zea mays	i embryo	Prioli and Sundahl, 1989
Zea mays	i embryo	Shillito et al., 1989

plant species: ^{i}=indica, ^{j}=japonica
explant type: i=immature, m=mature

2.8.2 *Protoplast culture*

Protoplasts of *Lolium* are cultured in liquid medium (Dalton, 1988AB, Creemers-Molenaar et al.,1989) or in agarose-solidified medium (Creemers-Molenaar et al.,1989) in the dark. As described for rice (Yamada et al.,1986) *Lolium* protoplasts also require a culture medium with a high osmolality (890-920 mOsm., mainly adjusted with glucose.
Although in *Lolium* the plating efficiency (expressed as the percentage of plated protoplasts that form microcalli) has been shown to be as high as 2.2% in liquid RY-2 medium, such values can only be achieved with protoplasts isolated from fast growing suspension cultures that have lost the potential for plant regeneration. Regeneration-competent suspension cultures yield few protoplasts ($<10^6$/g FW) with low plating efficiencies (0-0.1%). Furthermore, additional treatments like electro-fusion or PEG (polyethylene glycol) fusion have been shown to reduce the plating efficiency of protoplasts. In perennial ryegrass several modifications of the RY-2 medium improve the plating efficiency (Creemers-Molenaar et al.,1990,1991):
1) The addition of conditioned medium (50% V/V) to RY-2 medium improved the plating efficiency of protoplasts from aged suspension cultures and was indispensable for the proliferation of microcalli from protoplasts from young suspension cultures.
2) The addition of 10-30 mg/l ASA (O-acetylsalicylic acid) to conditioned RY-2 medium (RY-C) further enhanced the plating efficiency 1.5-3 times.
3) The addition of different combinations of antioxidants to the CPW-isolation and the RY-C culture medium improved the plating efficiency up to 5 times compared with protoplasts isolated and cultured without antioxidants (Creemers-Molenaar and van Oort, 1990).

2.8.3. *Plant regeneration from protoplasts*

Plant regeneration from *Lolium* protoplasts is obtained after several subcultures of compact callus to regeneration medium in the light (Creemers-Molenaar et al.,1989). Using this procedure soil-established plants have been regenerated from protoplasts of several suspension culture lines of *L. perenne*, representing 4 varieties. A gradual decrease of the 2,4-D concentration (RY-C4.4= conditioned RY-2 medium with 4.4 mg/l 2,4-D) in the successive regeneration media and a gradual adaptation to light conditions (RY-C4.4-dark, MS2.5-dark, MS0-indirect light, MS0-direct light) improved the proliferation of microcalli and plant regeneration frequency (Creemers-Molenaar, unpublished). The improved subculture regime, together with the use of conditioned RY-2 medium, resulted in the regeneration of 334 green plants per $3.5x10^5$ plated protoplasts.

2.9 **Production of haploid plants through anther culture**

Haploid green plants have been regenerated from anther culture in several gramineous species, including *L. perenne* and *L. multiflorum* (Table 2). Important progress was made by Olesen et al. (1988) who tested 65,745 anthers from 30 clones of 10 *L. perenne* varieties for callus induction and plant regeneration. The anthers containing microspores in the late uninucleate stage were cultured onto PII-substrate (Wang and Hu Han,1984) solidified with 0.3% (w/v) gelrite and supplemented with 2.5% (w/v) potato extract. For plant regeneration calli were transferred to regeneration medium 190-2 (Wang and Hu Han,1984) in the light. Twelve of the 30 clones, representing 5 varieties, produced anther-derived green plants.
It was shown that in *L. perenne* the genotype of the donor plants largely determined the response of cultured anthers with respect to callus induction and green plant regeneration (Olesen et al.,1988, Halberg et al.,1990, Bante et al.,1991). Recently, Halberg et al. (1990) showed that in *L. perenne*, clones with a superior anther culture response could be obtained through the sexual crossing of primary responsive clones. Next, these superior clones might be used as inducers of anther culture response through crossing with non-responding breeding material. Of course, a disadvantage of this approach is the need for several cycles of backcrossing.
In addition, attempts have been made to improve the anther culture response by modifying the culture conditions (Bante et al., 1990, 1991). The percentage anthers forming callus or embryos could be increased by: 1) substitution of sucrose by maltose, trehalose or maltotriose (2-6X), 2) reduction of the ammonium nitrate concentration in the induction medium (2X), 3) cold pretreatment of the spikes (4-5X) and, 4) incubation of the anthers in 2% CO2 (4-5X). When the former modifications were use in combination up to 35% of the anthers formed callus.
The application of anther culture in ryegrass breeding will only be successful if the method works well for a wide range of genotypes. The genotype-dependent response and the observation that the majority of the regenerated shoots are albinos make clear that further research is necessary.

2.10 **Albinism**

In tissue cultures of the Gramineae the incidence of albino shoots is often observed and can hinder progress in the development of genetic manipulation techniques. In *Lolium*, albino shoots are observed quite early after culture initiation and the frequency increases with culture age. Up to 42% of immature inflorescence-derived compact calli of *L. perenne* produced albino shoots (Creemers-Molenaar et al.,1988B). In *L. multiflorum* 50% of the shoots that

regenerated from embryo-derived callus cultures were albino (Dale,1980B). In anther culture of perennial ryegrass the majority (±80-100%) of the regenerated plants was albino (van Ark, personal communication).

The effect of aging on the occurrence of albino shoots is observed in callus cultures maintained on solid medium over prolonged periods (Dale et al.,1981), but is most pronounced in suspension cultures of *L. perenne* and *L. multiflorum* (Dalton,1988AB, Creemers-Molenaar et al.,1989). In addition to aging other factors have been reported to enhance the regeneration of albino shoots in *Lolium*:

1) higher culture temperature (Jackson and Dale,1988)
2) growth conditions of the donor plants, i.e. field conditions versus greenhouse conditions (Creemers-Molenaar et al.,1988B)
3) genotype (Olesen et al.,1988, Creemers-Molenaar et al.,1988B) and,
4) 2,4-D concentration of the culture medium (Creemers-Molenaar et al.,1988B).

Several of the above-mentioned factors have also been observed to influence the emergence of albino shoots in other gramineous species (Van der Valk et al., 1988,1989, Wang et al.,1978, Chandler and Vasil,1984, Lo et al.,1980).

A molecular characteristic of albinism, as demonstrated for wheat and barley albino plantlets, is the presence of large deletions in the chloroplast DNA (Day and Ellis, 1984,1985). In wheat anther culture it was shown that the inheritance of the response with respect to the percentage of green and albino shoots that regenerate, was dependent on the action of different classes of nuclear genes (Tuvesson et al., 1989). However, the cause of albinism and the possible involvement of nuclear and cytoplasmic genomes has not yet been elucidated.

2.11 **Progress in somatic hybridization and cybridization**

In gramineous species, sofar, the recovery of somatic hybrid and cybrid plants by protoplast fusion has only been successful in rice (*Oryza sativa* L.) or in fusions between cultivated rice and wild relatives or barnyard grass (Terada et al.,1987, Hayashi et al.,1988, Yang et al.,1988, Yang et al.,1989, Akagi et al.,1989). Recently, successful transfer of cytoplasmic male sterility to a fertile rice cultivar by electro-fusion has been reported by Kyozuka et al. (1989). As stated by the authors, recent progress in regenerating plants from protoplasts of rice has permitted the production of hybrid and cybrid plants.

In perennial ryegrass electro-fusion between suspension-derived protoplasts and mesophyl-derived protoplasts has been described (Creemers-Molenaar et al., 1988A). However, fused protoplasts did not divide and, subsequently, no microcalli have been obtained. Further experiments revealed that, compared with electrofusion, suspension culture derived

Table 5. Transgenic plants regenerated from gramineous species

Plant species	Trans. tissue	Trans. method	Gene(s)	References
rice (j)	pps	EP	*npt*II	Zhang et al. (1988)
rice[a] (j)	pps	PEG	*gus*	Zhang and Wu (1988)
rice (j)	pps	EP	*aph*II	Toriyama et al. (1988)
rice[a] (j)	pps	EP		Shimamoto et al. (1989)
rice[a] (j)	pps	PEG	*hpt*	Hayashimoto et al. (1990)
rice[a] (i)	pps	PEG	*hpt*	Datta et al. (1990)
orchardgrass	pps	PEG, EP	*aph*IV	Horn et al. (1988B)
maize	pps	EP	*npt*II	Rhodes et al. (1988)
maize[a]	susp.	MB	*bar*, *gus*	Gordon-Kamm et al. (1990)
maize[a]	susp.	MB	*bar*, *gus*, *als*, luciferase,	Fromm et al. (1990)

[a] Fertile transgenic plants regenerated

Abbreviations: **Plant species:** j: japonica species, i: indica species
Trans. tissue: transformed tissue, pps: protoplasts, susp:suspension culture
Trans. method: transformation method, EP: electroporation, PEG: polyethylene glycol, MB: microprojectile bombardment
Gene(s): *npt*: neomycin phosphotransferase gene, *gus*: glucuronidase gene, *aph*: aminoglycoside phosphotransferase gene, *hpt*: hygromycin B phosphotransferase gene, *bar*: phosphinothricin acetyltranferase gene, *als*: acetolactate synthase gene

protoplasts of perennial ryegrass showed better survival after polyethylene glycol (PEG) treatment. Using polyethylene glycol, gamma-irradiated protoplasts of a *L. perenne* CMS line were fused with iodoacetamide-inactivated protoplasts of a fertile *L. perenne* variety (Creemers-Molenaar, submitted for publication). From these fusion experiments cybrid calli were obtained that contained the nucleus of the recipient line and the mitochondrial genome of the donor line. Up until now, plant regeneration has not been achieved from these cybrid calli.

2.12 **Progress in transformation**

Transgenic gramineous plants have been obtained in rice, maize and orchardgrass (Table 5). Transformed plants in rice have only been obtained after direct DNA uptake by protoplasts. In maize, fertile transgenic plants have been obtained which stably express a gene for herbicide resistance. This was achieved by microprojectile bombardment of embryogenic suspension callus.
In *Lolium* transformed callus has been obtained after PEG treatment of suspension-derived protoplasts (Potrykus,1985). Until now, no transformed plants have been regenerated in any *Lolium* species.

3 **CONCLUSION**

The work that has been summarized in this Chapter clearly demonstrates that important progress has been made in the development of tissue culture techniques in *Lolium*. Individual plants, representing valuable genotypes, can routinely be cloned and stored *in vitro* by meristem tip culture. Successful plant regeneration from callus, suspension cultures, protoplasts and anther culture has been achieved in *L. perenne* and *L. multiflorum*. Furthermore, it has been demonstrated that cytoplasm of a male sterile *L. perenne* line can be transferred by asymmetric protoplast fusion to a fertile *L. perenne* variety. However, rapid loss of the regeneration potential of suspension cultures, the ocurrence of albinism and the limited number of genotypes/varieties able to regenerate plants from protoplasts and anther culture remain serious limitations to the widespread application of *in vitro* techniques in *Lolium* and need further investigation. In conclusion, although much work still has to be done, the achievements so far in the tissue culture of *Lolium* are most promising with respect to the future application of genetic manipulation techniques in plant breeding programs.

REFERENCES

Abdullah R, Cocking EC, Thompson JA (1986) Efficient plant regeneration from rice protoplasts through somatic embryogenesis. Bio/Technol 4:1087-1090

Ahloowalia BS (1975) Regeneration of ryegrass plants in tissue culture. Crop Science 15:449-452

Ahloowalia BS (1983) spectrum of variation in somaclones of triploid ryegrass. Crop Science 23:1141-1147

Ahuja PS, Pental D, Cocking EC (1982) Plant regeneration from leaf base callus and cell suspensions of *Triticum aestivum*. Z Pflanzenzucht 89:13-144

Akagi H, Sakamoto M, Negishi T, Fujimura T (1989) Construction of rice cybrid plants. Mol Gen Genet 215:501-506

Asano Y, Sugiura K (1990) Plant regeneration from suspension culture-derived protoplasts of *Agrostis alba* L. (Redtop). Plant Science 72:267-273

Atkin RK, Barton GE (1973) The establishment of tissue cultures of temperate grasses. J Exp Bot 24:689-699

Bajaj YPS (1989) Genetic engineering and in vitro manipulation of plant cells-technical advances. In Bajaj YPS (ed) Biotechnology in agriculture and forestry, vol 9: Plant protoplasts and genetic engineering II. Springer, Berlin Heidelberg New York Tokyo, pp 1-25

Bante I, Sonke T, Tandler RF, van den Bruel AM, Meijer EM (1990) Anther culture of *Lolium perenne* L. and *Lolium multiflorum* L. Acta Bot. Neerl. 39(1):103

Bante I, Sonke T, Tandler RF, van den Bruel AM, Meijer EM (1991) Anther culture of *Lolium perenne* and *Lolium multiflorum*. In: Sangwan BS and Sangwan RS (eds.), The impact of biotechnology in agriculture. International Conference, Amiens, France 10-12 July 1989 (in press)

Binh DQ, Heszky LE (1990) Restoration of the regeneration potential of long-term culture in rice (*Oryza sativa* L.) by salt pretreatment J Plant Physiol 136:336-340

Boppenmeier J, Zuchner S, Foroughi-Wehr B (1989) Haploid production from barley yellow dwarf virus resistant clones of *Lolium*. Plant Breeding 103:216-220

Breese EL, Tyler BF (1986) Patterns of variation and the underlying genetic and cytological architecture in grasses with particular reference to *Lolium*. In: Styles BT (ed) Intraspecific classification of wild and cultivated plants. Clarendon, Oxford pp 53-69

Chandler SF, Vasil IK (1984). Optimization of plant regeneration from long term embryogenic callus cultures of *Pennisetum purpureum* grass). J Plant Physiol 117:147-156

Chen WH, Davey MR, Power JB, Cocking EC (1988) Sugarcane protoplasts:Factors affecting division and plant regeneration. Plant Cell Rep 7:344-347

Conger BV, Carabia JV, Lowe KW (1978) Comparison of 2,4-D and 2,4,5-T on callus induction and growth in three Gramineae species. Environ Exp Bot 18:163-168

Conger BV, Hilenski LL, Lowe KW, Carabia JV (1982) Influence of different auxins at varying concentrations on callus induction and growth from embryo and leaf-tip explants in Gramineae. Environ Exp Bot 22:39-48

Creemers-Molenaar J, Loeffen JPM, Zaal MACM (1988a) Protoplasts from *Lolium perenne* L.: Procedures for plant regeneration and electro-fusion. Proceedings of the second International Congress of Plant Molecular Biology. Jerusalem. 13-18 Nov. p. 430

Creemers-Molenaar J, Loeffen JPM, Van der Valk P (1988b) The effect of 2,4-Dichlorophenoxyacetic acid and donor plant environment on plant regeneration from immature inflorescence-derived callus of *Lolium perenne* L. and *multiflorum* L. Plant Sci 57:165-172

Creemers-Molenaar J, Van der Valk P, Loeffen JPM, Zaal MACM (1989) Plant regeneration from suspension cultures and protoplasts of *Lolium perenne* L. Plant Sci 63:167-176

Creemers-Molenaar J, Van Oort Y (1990) Antioxidants influence plating efficiency and microcallus growth of protoplasts in *Lolium perenne* L. In: H.J.J. Nijkamp, L.H.W. van der Plas and J. van Aartrijk (Eds.): Progress in plant cellular and molecular biology, Proceedings VIIth International Congress on Plant Tissue and Cell Culture. Kluwer Academic Publishers, Amsterdam. pp 44-49.

Creemers-Molenaar J (1991) Regeneration from protoplasts of perennial ryegrass; progress and applications. In: den Nijs APM and Elgersma A (eds.), Fodder crops breeding: achievements, novel strategies and biotechnology. Proceedings of the 16th meeting of the Fodder Crops Section of Eucarpia, Wageningen, The Netherlands 16-22 November 1990 pp 123-128

Dale PJ (1975) Meristem tip culture in *Lolium multiflorum*. J Exp Bot 26:731-736

Dale PJ (1977a) Meristem tip culture in *Lolium, Festuca, Phleum* and *Dactylis*. Plant Sci Lett 9:333-338

Dale PJ (1977b) The elimination of ryegrass mosaic virus from *Lolium multiflorum* by meristem tip culture. Ann Appl Biol 85:93-96

Dale PJ (1980a) A method for *in vitro* storage of *Lolium multiflorum* Lam. Ann Bot 45:497-502

Dale PJ (1980b) Embryoids from cultured immature embryos of *Lolium multiflorum*. Z Pflanzenphysiol 100:73-77

Dale PJ, Thomas E, Brettell RIS, Wernicke W (1981) Embryogenesis from cultured immature inflorescences and nodes of *Lolium multiflorum*. Plant Cell Tissue Organ Culture 1:47-55

Dale PJ, Dalton SJ (1983) Immature inflorescence culture in *Lolium, Festuca, Phleum* and *Dactylis*. Z Pflanzenphysiol 111:39-45

Dalton SJ (1988a) Plant regeneration from cell suspension protoplasts of *Festuca arundinacea* Schreb. (tall fescue) and *Lolium perenne* L. (perennial ryegrass). J Plant

Physiol 132:170-175
Dalton SJ (1988b) Plant regeneration from cell suspension protoplasts of *Festuca arundinacea* Schreb., *Lolium perenne* L. and *L. multiflorum* Lam. Plant Cell Tissue Organ Culture 12:137-140
Datta SK, Peterhans A, Datta K, Potrykus I (1990) Genetically engineered indica-rice recovered from protoplasts. Bio/Technology 8:736-740
Day A, Ellis THN (1984) Chloroplast DNA deletions associated with wheat plants regenerated from pollen: possible basis for maternal inheritance of chloroplasts. Cell 39:359-368
Day A, Ellis THN (1985) Deleted forms of plastid DNA in albino plants from cereal anther culture. Curr Genet 9:671-678
Dudits D, Nemet G, Haydu Z (1975) Study of callus growth and organ formation in wheat (*Triticum aestivum*) tissue cultures. Can J Bot 53:957-963
Fitch, MMM, Moore, PH (1990) Comparison of 2,4-D and picloram for selection of long-term totipotent green callus cultures of sugarcane. Plant Cell Tissue and Organ Culture 20:157-163
Frakes RV (1973) The ryegrasses. In: Heath ME, Metcalfe DS, Barnes RF (eds) Forages 3rd edn Iowa State Univ Press, Ames, Iowa pp 307-313
Frandsen HN Frandsen KJ (1948) Polycross-metoden. Nord Jordbruksforsk 7-8:239-261
Frearson EM, Power JB, Cocking EC (1973) The isolation, culture and regeneration of *Petunia* leaf protoplasts. Dev Biol 33:130-137
Fromm ME, Morrish F, Armstrong C, Williams R, Thomas J, Klein TM (1990) Inheritance and expression of chimeric genes in the progeny of transgenic maize plants. Bio/Technology 8:833-839
Glimelius K, Fahlesson J, Landgren M, Sjodin C, Sundberg E (1991) Gene transfer via somatic hybridization in plants. TibTech 9:24-30
Gordon-Kamm WJ, Spencer TM, Mangano ML, Adams TR, Daines RJ, Start WG, O'Brien JV, Chambers SA, Adams WR, Willetts, NG, Rice TB, Mackey CJ, Krueger RW, Kausch AP, Lemaux PG (1990) Transformation of maize cells and regeneration of fertile transgenic plants. The Plant Cell 2:603-618
Gray DJ, Conger BV, Hanning GE (1984) Somatic embryogenesis in suspension and suspension-derived callus cultures of *Dactylis glomerata*. Protoplasma 122:196-202
Halberg N, Olesen A, Tuvesson IKD, Andersen SB (1990) Genotypes of perennial ryegrass (*Lolium perenne* L.) with high anther-culture response through hybridization. Plant Breeding 105:89-94
Hallauer AR, Miranda JB (1981) Selection: theory. In: Hallauer AR, Miranda JB (eds) Quantitative genetics in maize breeding. Iowa State Univ Press, Ames, Iowa, pp 159-204
Harris R, Wright M, Byrne M, Varnum J, Brightwell B, Schubert K (1988) Callus formation and plantlet regeneration from

protoplasts derived from suspension cultures of wheat (*Triticum aestivum* L.). Plant Cell Rep 7:337-340

Hayashi Y, Kyozuka J, Shimamoto K (1988) Hybrids of rice (*Oryza sativa* L.) and wild *Oryza* species obtained by cell-fusion. Mol Gen Genet 214:6-10

Hayashimoto A, Li Z, Murai N (1990) A polyethylene glycol-mediated protoplast transformation system for production of fertile transgenic rice plants. Plant Physiol 93:857-863

Hayward MD, Abdullah IB (1985) Selection and stability of synthetic varieties of *Lolium perenne* L. The selected character and its expression over generations of multiplication. Theor Appl Genet 70: 48-51

Hayward MD, Olesen A, Due IK, Jenkins R, Morris P (1990) Segregation of isozyme marker loci amongst androgenetic plants of *Lolium perenne* L. Plant Breeding 104:68-71

Ho W, Vasil IK (1983) Somatic embryogenesis in sugarcane (*Saccharum officinarum* L.): Growth and plant regeneration from embryogenic cell suspension cultures. Ann Bot 51:719-726

Horn ME, Conger BV, Harms CT (1988A) Plant regeneration from protoplasts of embryogenic suspension cultures of orchardgrass (*Dactylis glomerata* L.). Plant Cell Rep 7:371-374

Horn ME, Shillito RD, Conger BV, Harms CT (1988B) Transgenic plants of orchardgrass (*Dactylis glomerata* L.) from protoplasts. Plant Cell Rep 7:469-472

Jackson JA, Dalton SJ, Dale PJ (1986) Plant regeneration from root callus in the forage grass *Lolium multiflorum* In: Withers LA, Alderson PJ (eds) Plant tissue culture and its agricultural applications. Butterworths, London pp 85-89

Jackson JA, Dale PJ (1988) Callus induction, plant regeneration and an assessment of cytological variation in regenerated plants of *Lolium multiflorum* L. J Plant Physiol 132:351-355

Joarder OI, Joarder NH, Dale PJ (1986) In vitro response of leaf tissues from *Lolium multiflorum*-a comparison with leaf segment position, leaf age and *in vivo* mitotic activity. Theor Appl Genet 73:286-291

Jones MB, Lazenby A (1988) Preface. In: Jones and Lazenby (eds) The Grass Crop. Chapman and Hall Ltd., London, New York pp 277-309

Jones MGK, Dale PJ (1982) Reproducible regeneration of callus from suspension culture protoplasts of the grass *Lolium multiflorum*. Z Pflanzenphysiol 105:267-274

Jonsson HA, Nilsson C (1986) Plant breeding for resistance to snow moulds in grasses. Nord Jordbruksforsk 68:596

Kamo KK, Hodges TK (1986) Establishment and characterization of long-term embryogenic maize callus and cell suspension cultures. Plant Sci 45:111-117

Kasperbauer MJ, Buckner RC, Bush LP (1979) Tissue culture of annual ryegrass x tall fescue F_1 hybrids: Callus establishment and plant regeneration. Crop Sci 19:457-460

Kyozuka J, Hayashi Y, Shimamoto K (1987) High frequency plant regeneration from rice protoplasts by novel nurse culture methods. Mol Gen Genet 206:408-413

Kyozuka J, Otoo E, Shimamoto K (1988) Plant regeneration from protoplasts of indica rice: genotypic differences in culture response. Theor Appl Genet 76:887-890

Kyozuka J, Kaneda T, Shimamoto K (1989) Production of cytoplasmic sterile rice (*Oryza sativa* L.) by cell fusion. Bio/Technology 7:1171-1174

Lee L, Schroll RE, Grimes HD, Hodges TK (1989) Plant regeneration from indica rice (*Oryza sativa* L.) protoplasts. Planta 178:325-333

Linsmaier EM, Skoog F (1965) Organic growth factor requirements of tobacco tissue cultures. Physiol Plant 18:100-127

Lo PF, Chen CH, Ross JG (1980) Vegetative propagation of temperate forage grasses through callus culture. Crop Sci 20:363-367

Lu C, Vasil V, Vasil IK (1981) Isolation and culture of protoplasts of *Panicum maximum* Jacq. (guinea grass): Somatic embryogenesis and plantlet formation. Z Pflanzenphysiol 104:311-318

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473-497

Niizeki M (1977) Haploid, polyploid and aneuploid plants from cultured anthers and calluses in species of *Nicotiana* and forage crops. J Faculty Agric 58:343-466

Nitzsche W, Wenzel G (1977) Haploids in plant breeding.In: Fortschritte der Pflanzenzuchtung 8. Advances in plant breeding. Parey, Berlin, pp 46-48

Olesen A, Andersen SB, Due IK (1988) Anther culture in perennial ryegrass (*Lolium perenne* L.). Plant Breeding 101:60-65

Orshinsky BR, Mcgregor LJ, Johnson GIE, Hucl P, Kartha K (1990) Improved embryoid induction and green shoot regeneration from wheat anthers cultured in medium with maltose. Plant Cell Rep 9: 365-369

Pagniez M, Demarly Y (1979) Obtention d'individus androgenetiques par culture in vitro d'antheres de Ray-grass d'Italie (*Lolium multiflorum* Lam.). Ann Amelior Plantes 29:631-637

Plumb RT (1988) The effects of pests and diseases on grasses. In: Jones MB and Lazenby A (eds) The Grass Crop. Chapman and Hall Ltd., London, New York pp 277-309

Potrykus I, Saul MW, Petruska W, Paszkowski J, Shillito RD (1985) Direct gene transfer to cells of a gramineceous monocot. Molec Gen Genet 199:183-188

Potrykus I (1990) Gene transfer to cereals: an assessment. Bio/Technol 9:535-542

Prioli LM, Sondahl MR (1989) Plant regeneration and recovery of fertile plants from protoplasts of maize (*Zea mays* L.).

Bio/technoloy 7:589-594
Rajoelina SR, Alibert G, Planchon C (1990) Continuous plant regeneration from established embryogenic cell suspension cultures of Italian ryegrass and tall fescue. Plant Breeding 104:265-271
Rhodes CA Lowe KS, Ruby KL (1988a) Plant regeneration from protoplasts isolated from embryogenic maize cell cultures. Bio/Technology 6:56-60
Rhodes CA, Pierce DA, Mettler IJ, Mascarenhas D, Detmer JJ (1988b) Genetically transformed maize plants from protoplasts. Science 240:204-206
Rose JB, Dunwell JM, Sunderland N (1987) Anther culture of *Lolium temulentum*, *Festuca pratensis* and *Lolium* x *Festuca* hybrids. I. Influence of pretreatment, culture medium and culture incubation conditions on callus production and differentiation. Ann Bot 60:191-201
Schaepman H (1952) Application of the polycross test to grass breeding. Euphytica 1:105-111
Schmidt F, Posselt UK (1990) Reproducable regeneration of callus cultures derived from mature embryos in *Lolium perenne*. In: den Nijs APM and Elgersma A (eds.), Fodder crops breeding: achievements, novel strategies and biotechnology. Proceedings of the 16th meeting of the Fodder Crops Section of Eucarpia, Wageningen, The Netherlands 16-22 November 1990 pp 205-206
Schmidt F (1991) Entwicklung eines Gewebekultursystems bei *Lolium perenne* (L.) als Voraussetzung zur *in vitro*-Resistenzselektion. Ph. D. thesis, Universitat Hohenheim.
Shillito RD, Carswell GK, Johnson CM, DiMaio JJ, Harms CT (1989) Regeneration of fertile plants from protoplasts of elite inbred maize. Bio/Technology 7:581-587
Shimamoto K, Terada R, Izawa T, Fujimoto H (1989) Fertile transgenic rice plants regenerated from transformed protoplasts. Nature 338:274-276
Skene KGM, Barlass M (1983) Regeneration of plants from callus cultures of *Lolium rigidum*. Z Pflanzenzucht 90:130-135
Srinivasan C, Vasil IK (1986) Plant regeneration from protoplasts of sugarcane (*Saccharum officinarum* L.). J Plant Physiol 126:41-48
Stanis VA, Butenko RG (1984) Developing viable haploid plants in anther culture of ryegrass. Dokly Biol Sci 275:249-251
Strickland SG, Nichol JW, McCall CM, Stuart DA (1987) Effect of carbohydrate source on alfalfa somatic embryogenesis. Plant Science 48:113-121
Takamizo T, Suginobo K, Ohsugi R (1990) Plant regeneration from suspension culture derived protoplasts of tall fescue (*Festuca arundinacea* Schreb.) of a single genotype. Plant Science 72:125-131
Terada R, Kyozuka J, Nishibayashi S, Shimamoto K (1987) Plantlet regeneration from somatic hybrids of rice (*Oryza sativa* L.) and barnyard grass (*Echinochloa oryzicola* Vasing). Mol Gen Genet 210:39-43

Torello WA, Mancino L, Troll J (1983) Initiation, growth, and maintenance of callus tissue derived from mature caryopses of perennial ryegrass *(Lolium perenne* L.). Rasen Turf Gazon 1:4-7

Torello WA, Symington AG (1984) Regeneration from perennial ryegrass callus tissue. Hortic Sci 19:56-57

Toriyama K, Hinata K, Sasaki T (1986) Haploid and diploid plant regeneration from protoplasts of anther callus in rice. Theor Appl Genet 73:16-19

Toriyama K, Arimoto Y, Uchimiya H, Hinata K (1988) Transgenic rice plants after direct gene transfer into protoplasts (1988) Bio/Technology 6:1072-1074

Tuvesson IKD, Pedersen S, Andersen SB (1989) Nuclear genes affecting albinism in wheat (*Triticum aestivum* L.) anther culture. Theor Appl Genet 78:879-883

Tyler BF (1987) Description and distribution of natural variation in forage grasses In:INRA (eds) Proc of the Eucarpia fodder crops section meeting. Lusignan pp 13-22

Utz HF Oettler G (1978) Performance of inbred lines and their top crosses in perennial ryegrass (*Lolium perenne* L). Pflanzenzuchtung 80:223-229

Van der Valk P, Zaal MACM, Creemers-Molenaar J (1988) Regeneration of albino plantlets from suspension culture derived protoplasts of *Poa pratensis* L. (Kentucky bluegrass). Euphytica Suppl:169-176

Van der Valk P, Zaal MACM, Creemers-Molenaar J (1989) Callus induction and plant regeneration from immature inflorescences and mature seeds of *Poa pratensis* L. (Kentucky bluegrass). Plant Cell Rep 7:644-647

Vasil V, Vasil IK (1986) Plant regeneration from friable embryogenic callus and cell suspension cultures of *Zea mays* L. J Plant Physiol 124:399-408

Vasil IK (1987) Developing cell and tissue culture systems for the improvement of cereal and grass crops. J Plant Physiol 128:193-218

Vasil IK (1988) Progress in the regeneration and the genetic manipulation of cereal crops. Bio/Technology 6:397-402

Vasil V, Redway F, Vasil IK (1990A) Regeneration of plants from embryogenic suspension culture protoplasts of wheat (*Triticum aestivum* L.). Bio/Technology 8:429-434

Vasil IK (1990B) The realities and challenges of plant biotechnology. Bio/Technol 8:296-301

Wang C, Sun C, Chu C, Wu S (1978) Studies on the albino pollen plantlets in rice. In: Proceedings of Symposium on Plant Tissue Culture. Pittman, Peking, pp 149-160

Wang D, Miller PD, Sondahl MR (1989) Plant regeneration from protoplasts of Indica type rice and CMS rice. Plant Cell Rep 8:329-332

Wang WC, Nguyen HT (1990) A novel approach for efficient plant regeneration from long-term suspension culture of wheat. Plant Cell Rep 8:639-642

Wang X, Hu Han (1984) The effect of potato II medium for

Triticale anther culture. Plant Sci Lett 36:237-239

Wit F (1959) Chromosome doubling and the improvement of grasses. Agraria 11:97-115

Yamada Y, Zhi-Qi Y, Ding-Tai T (1986) Plant regeneration from protoplast-derived callus of rice (*Oryza sativa* L.). Plant Cell Rep 5:85-88

Yang Z-Q, Shikanai T, Yamada Y (1988) Asymmetric hybrization between cytoplasmic male-sterile and fertile rice (*Oryza sativa* L.) protoplasts. Theor Appl Genet 76:801-808

Yang Z-Q, Shikanai T, Yamada Y (1989) Plant regeneration from cytoplasmic hybrids of rice (*Oryza sativa* L.). Theor Appl Genet 77:305-310

Yasuda T, Miyano S, Yamamoto Y, Uchida N, Yamaguchi, T (1990) Analogues of phenoxyacetic acid and the generation of calluses from seeds of indica rice. Plant Cell Physiol 31(6):763-766

Zaghmout OMF, Torello WA (1989) Somatic embryogenesis and plant regeneration from suspension cultures of red fescue. Crop Sci 29:815-817

Zaghmout OMF, Torello WA (1990a) Isolation and culture of protoplasts from embryogenic suspension cultures of red fescue (*Festuca rubra* L.). Plant Cell Rep 9:340-343

Zaghmout OMF, Torello WA (1990b) Somatic embryogenesis and plant regeneration from embryogenic suspension cultures of perennial ryegrass. In Vitro Cell Dev Biol 26:419-424

Zhang HM, Yang H, Rech EL, Golds TJ, Davis AS, Mulligan BJ, Cocking EC, Davey MR (1988) Transgenic rice plants produced by electroporation-mediated plasmid uptake into protoplasts. Plant Cell Rep 7:379-384

Zhang W, Wu R (1988) Efficient regeneration of transgenic plants from rice protoplasts and correctly regulated expression of the foreign gene in the plants. Theor Appl Genet 76:835-840

Zimny J, Lörz H (1986) Plant regeneration and initiation of cell suspensions from root-tip derived callus of *Oryza sativa* L. Plant Cell Rep 5:89-92

CHAPTER 2

THE EFFECT OF 2,4-DICHLOROPHENOXYACETIC ACID AND DONOR PLANT ENVIRONMENT ON PLANT REGENERATION FROM IMMATURE INFLORESCENCE-DERIVED CALLUS OF *LOLIUM PERENNE* L. AND *LOLIUM MULTIFLORUM* L.

With: J. P. M. Loeffen and P. van der Valk

Plant Science (1988) 57: 165-172

SUMMARY

Callus cultures were obtained from immature inflorescences of perennial ryegrass (*Lolium perenne*) and italian ryegrass (*Lolium* multiflorum). Inflorescence segments were cultured on Murashige and Skoog medium (MS) supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D). The response in culture with regard to compact callus induction, embryogenesis and plant regeneration was determined for different varieties. The in vitro response was compared for explants from field-grown plants and explants from greenhouse-grown plants. The effect of different 2,4-D concentrations on the in vitro response was also investigated in one *L. perenne* variety and one *L. multiflorum* variety.

The percentage of explants that formed compact callus and embryogenic callus differed strongly with the cultivar. There was no unanimous effect of the growth conditions of the donor plants or the 2,4-D concentration of the medium on this response. Green plants were regenerated from all the cultivars tested. Explants from field-grown plants showed a higher tendency to form albino shoots than explants from greenhouse-grown plants. In the *L. perenne* variety tested higher 2,4-D concentrations (up to 15 mg/l) resulted in a lower regeneration frequency of green shoots and a higher regeneration frequency of albino shoots (up to 12.5 mg/l). In the *L. multiflorum* variety tested the effect of 2,4-D on regeneration was less pronounced.

INTRODUCTION

The application of novel genetic manipulation techniques in plant breeding, such as somatic hybridization and direct gene transfer, requires the availability of a reliable system for plant regeneration from protoplasts and single cells. In the Gramineae protoplasts can be obtained directly from all plant parts, but the culture of these protoplasts has not yet been successful. The alternative is to culture explants and to use proliferating morphogenic callus either directly, or after the establishment of a morphogenic cell suspension culture, for protoplast isolation. Plant regeneration from cell suspension-derived protoplasts has been reported for rice (Thompson et al., 1986, Yamada et al., 1986), sugarcane (Srinivasan and Vasil, 1986), italian ryegrass and tall fescue (Dalton, 1988). These suspension cultures were established from embryogenic callus initiated from immature embryo explants.

In *L. perenne*, plant regeneration was reported from embryogenic callus initiated on mature caryopses (Torello et al., 1983, Torello and Symington, 1984) and immature inflorescences (Dale and Dalton, 1983). In the latter case shoots originated from floral primordia rather than from callus and no embryogenesis was observed. In *L. multiflorum*, plant regeneration has been achieved from embryogenic callus initiated on immature embryos (Dale, 1980) and immature inflorescences (Dale et al., 1981). Callus without competence for morphogenesis (Jones and Dale, 1982), as well as green plants (Dalton, 1988), were obtained from cell suspension-derived protoplasts of *L. multiflorum*.

In order to develop a system for somatic hybridization and transformation of *L. perenne* and *L. multiflorum* our first aim was to establish a reliable method for plant regeneration from callus. In the present study the in vitro response of immature inflorescences of different *L. perenne* and *L. multiflorum* varieties was analysed with respect to callus initiation, embryogenesis and plant regeneration. Since the availability of immature inflorescences under field conditions is limited by a fixed, short flowering period, greenhouse-grown plants were considered as a good alternative. However, for maize embryos (Lu et al., 1983, Santos and Torne, 1986) it has been reported that callus induction and callus growth is influenced by the growth conditions of the donor plants. Therefore, the response of field-grown plants was compared with that of vernalized greenhouse-grown plants. To determine the optimal 2,4-D level for callus induction and plant regeneration, explants from one *L. perenne* and one *L. multiflorum* variety were cultured at different 2,4-D concentrations.

MATERIALS AND METHODS

Immature inflorescences were obtained from field- and greenhouse-grown plants of different *Lolium perenne* and *Lolium multiflorum* varieties. Greenhouse-grown plants were induced to flower during the winter period by artificial vernalization of seedlings as described by Bühring (1977).

Tillers containing unemerged inflorescences were collected at random from different plants of each variety and surface sterilized in 70% ethanol for 2 s, followed by 5% (w/v) sodium hypochlorite plus 2 drops Tween 80 per 100 ml for 10 minutes. After rinsing 5 times in sterile water, 4-7 mm long immature inflorescences were excised from the tillers and the basal parts of each were cut into 2 segments (1-2 mm long). For callus initiation and proliferation 6 segments were cultured per Petri dish (Greiner, 94x16mm) on Murashige and Skoog medium (1962) supplemented with (per liter) 8 g agar, 30 g sucrose, 0.4 mg thiamine-HCl and 5 mg 2,4-D at pH 5.8 (MS5, 5 referring to the 2,4-D concentration). All media components were filter sterilized, except for agar which was autoclaved as a concentrated solution for 15 min at 121°C. Different 2,4-D concentrations were used as indicated in the text.

Callus was initiated during a culture period of 4 weeks in the dark at 25°C. After 3 two-weekly subcultures on the same medium, compact callus was successively subcultured on MS0.2 in the dark for 3 weeks and on MS0 in the light for 4 weeks. The light regime consisted of continuous light from cool white fluorescent lamps at approximately 7000 lux at 25°C. Shoots were transferred to culture vessels with half strength MS0 and green plantlets with roots were potted in soil.

Frequencies of callus induction, embryogenesis and plant regeneration were calculated per explant and not per inflorescence. For embryogenesis and plant regeneration the

response of only those explants that formed compact callus was analysed. A subdivision was made between explants that regenerated only green shoots and explants that regenerated albino shoots with or without green shoots. To what extent the frequency of compact and embryogenic callus formation depended on the cultivar or the growth conditions was analysed using a generalized linear model based on a binomial distribution and with a logit as link function (McCullag and Nelder, 1983). The relation between the percentage of compact calli regenerating green or albino shoots, the cultivar and the growth conditions was modelled using a multinomial model (McCullag and Nelder, 1983). All statistical analyses were done with the package/language Genstat.

RESULTS

Callus initiation and regeneration.

Immature inflorescence segments from 12 *L.perenne* and 5 *L.multiflorum* varieties were tested for callus initiation, embryogenesis and plant regeneration. Although differences were observed between varieties with respect to the quantity of different callus types that proliferated and the number of shoots that regenerated, the in vitro response for all tested varieties can be described as follows. After incubation for 2 to 4 weeks on MS5 the inflorescence explants formed a watery type of callus. At the end of the first culture period or after 1 to 3 two-weekly subcultures on the same medium centres of compact callus were formed in this watery callus (Fig. 1a). Compact callus was pale yellow and was either rough and friable or smooth and globular. Differentiation of callus into roots and shoots often occurred during the callus initiation period on MS-medium with 5 mg/l 2,4-D (Fig. 1a). When pieces of compact callus were transferred to MS0.2 pro-embryogenic structures and mature somatic embryos could be observed within 3 weeks on some of the explants (Fig.1b). As described for *Pennisetum purpureum* (Wang and Vasil, 1982) and *Triticum aestivum* (Ozias-Akins and Vasil, 1982), abnormal development of embryoids, resembling fused cup-shaped scutella (Fig.1c), often occurred.

When pieces of compact callus were subcultured from MS0.2 to MS0 in the light some of the calli turned brown or formed only roots. The other calli regenerated 1) green shoots (Fig. 1d), 2) green and albino shoots or 3) albino shoots only. Compact callus pieces derived from the same explant often differed with respect to the type of shoots which they produced. The process of shoot regeneration occurred either through germination of somatic embryos (embryogenesis) or directly from the callus (organogenesis). In many cases germinating somatic embryos formed a shoot without visible root formation, thus resembling organogenesis. From all the cultivars tested green shoots could

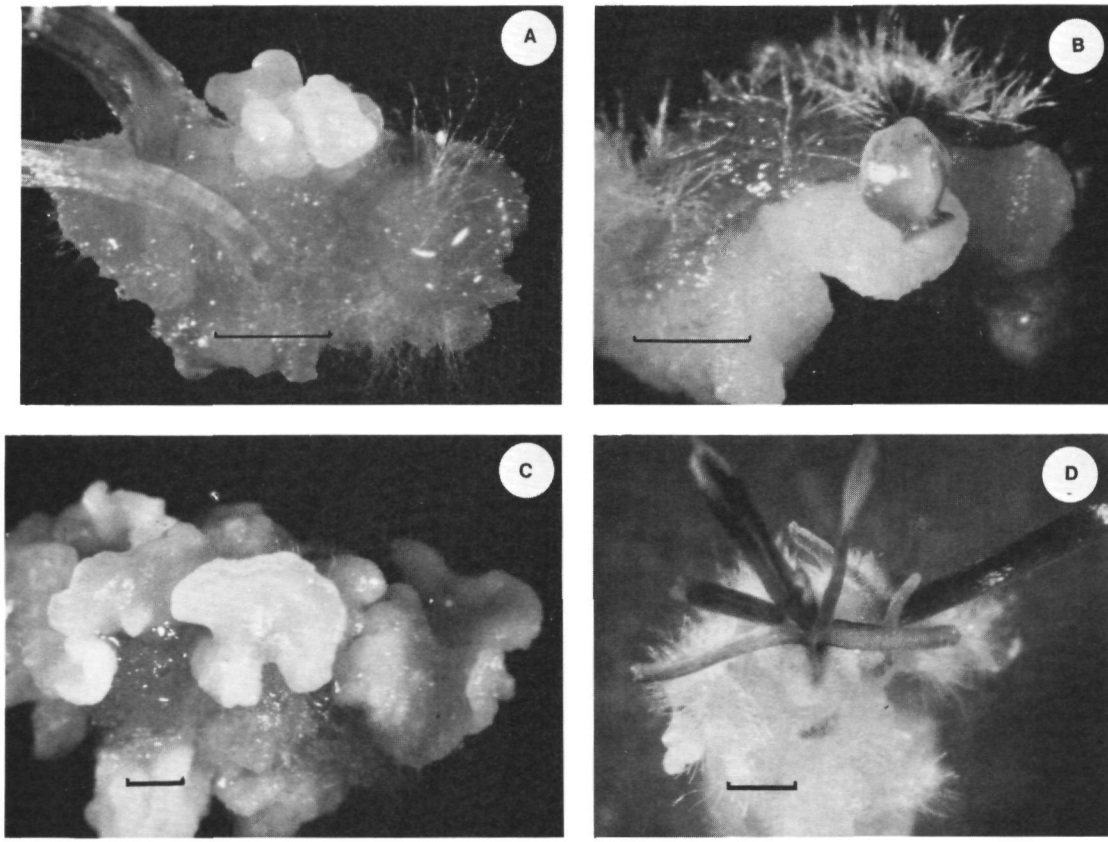

Fig. 1. Callus initiation and regeneration from immature inflorescence explants of *L. perenne* and *L. multiflorum*. (a) Watery callus with compact callus. (b) Well developed somatic embryo. (c) Atypical somatic embryogenesis. (d) Regeneration of green shoots from compact callus. Scale: bar=1 mm.

be regenerated. Green shoots were transferred to half strength MS0 in culture vessels to stimulate root growth. The plantlets were then potted in soil and grown to phenotypically normal mature plants.

Effects of growth conditions of the donor plants on callus initiation and regeneration.

The response in culture of immature inflorescence explants from 4 *L.perenne* and 2 *L.multiflorum* varieties grown under different growth conditions has been determined. Table 1 shows that 54% to 100% of the explants from greenhouse-grown plants and 31% to 83% of the explants from field-grown plants formed compact callus. Embryogenic callus was formed from 8% to 100% of the compact calli derived from greenhouse-grown plants and from 22% to 53% of the compact calli derived from field-grown plants. The frequency of compact and embryogenic callus formation varied with the cultivar and the growth conditions of the donor plants; statistical analysis showed a substantial interaction ($p<<0.001$) between the effect of the growth conditions and the cultivar. The effect of the growth conditions was most pronounced in varieties Lp9 and Lm1. Here, the frequency of explants forming compact callus was doubled when the donor plants were grown under greenhouse conditions instead of field conditions.

Fig. 2a shows that the regeneration frequency of green shoots varied with the growth conditions and the cultivar; again there was significant interaction ($p<0.01$) between both effects. Pronounced differences were again observed for variety Lp9. In Lp9 the percentage of explants forming only green shoots was 57% for field-grown plants and 96% for greenhouse-grown plants.

The frequency of explants that formed albino shoots is shown in Fig. 2b. In the 6 varieties tested the percentage of explants that formed albino shoots was higher for explants derived from field-grown plants than from greenhouse-grown plants. Statistical analysis showed a significantly ($p<0.001$) higher regeneration frequency of albino shoots under field conditions and no interaction with the cultivar. The tendency to form albino shoots differed with the cultivar: Lp13, a tetraploid variety, regenerated albino shoots at low frequency (0-3%), while in variety Lp14, a diploid, albino shoots were regenerated from 23% to 42% of the explants.

Callus cultures from explants of field-grown plants of 3 varieties were maintained for about one year by selective subculture of compact callus (17-21 transfers) on MS5. After this time, callus cultures from Lp9 still regenerated green shoots, Lp1 only white shoots and Lm10 no shoots at all.

Table 1. Callus formation from young inflorescence segments of *L.perenne* and *L multiflorum*. Figures in parentheses are numbers of explants tested. G=greenhouse grown plants, F=field grown plants, 2n=diploid, 4n=tetraploid.

variety	growth conditions	% explants with compact callus[a]	% embryogenic calli[b]
Lp1 2n	G	58 (24)	36 (14)
	F	67 (48)	22 (32)
Lp9 2n	G	100 (24)	100 (24)
	F	56 (48)	52 (27)
Lp14 2n	G	58 (24)	36 (14)
	F	63 (48)	50 (24)
Lp13 4n	G	63 (24)	73 (15)
	F	83 (48)	53 (38)
Lm11 2n	G	71 (24)	24 (17)
	F	31 (48)	33 (12)
Lm10 4n	G	54 (24)	8 (13)
	F	50 (48)	30 (23)

[a] Observations were made after 3 subcultures on the same medium.
[b] Observations were made after subculture to MS0.2 of 3 pieces of compact callus (5x5mm) per explant.

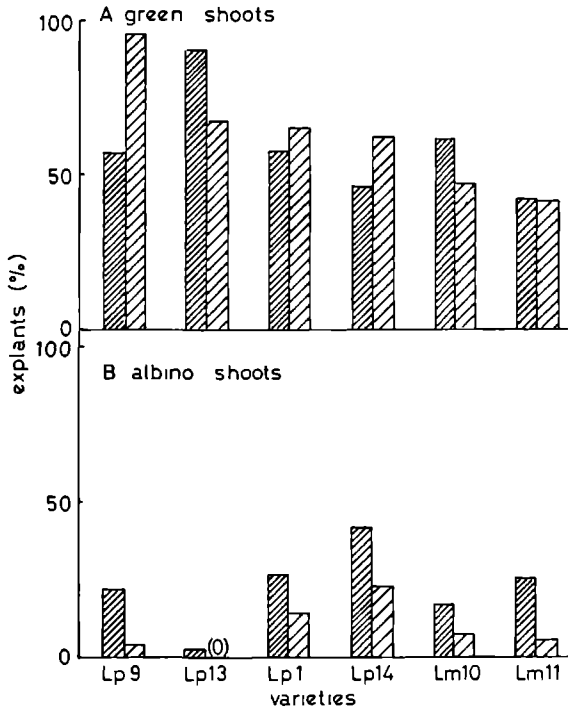

Fig. 2. Regeneration frequency of immature inflorescence explants in dependence of the growth conditions of the donor plants in *L.perenne* and *L.multiflorum*. The percentages are the means of 12 to 38 explants with compact callus. Observations were made after subculture of 3 pieces of compact callus per explant.
greenhouse-grown
field-grown plants

Table 2. The effect of different 2,4-D concentrations on callus formation and somatic embryogenesis from immature inflorescence segments of *L. perenne* and *L. multiflorum*. Figures in parenthesis are numbers of explants tested.

variety	2,4-D (mg/l)	% explants with compact callus[a]	% embryogenic calli[b]
Lp1	2.5	71(24)	27(15)
	5.0	58(24)	36(14)
	7.5	67(24)	40(15)
	10.0	63(24)	33(15)
	12.5	75(24)	44(18)
	15.0	75(24)	44(18)
Lm2	2.5	29(24)	71(7)
	5.0	42(26)	63(8)
	7.5	58(24)	57(14)
	10.0	48(23)	50(8)
	12.5	48(23)	44(9)
	15.0	38(24)	44(9)

[a] Observations were made after 3 subcultures on the same medium.
[b] Observations were made after subculture to MS0.2 of 3 pieces of compact callus (5x5mm) per explant.

Effects of 2,4-D on callus induction and regeneration.

The effect of different 2,4-D concentrations on callus initiation and regeneration was investigated in one *L. perenne* (Lp1) and one *L. multiflorum* (Lm2) variety. Table 2 shows that 58% to 75% of the explants from Lp1 formed compact callus. In Lm2 29% to 58% of the explants formed compact callus. The frequency of compact and embryogenic callus formation was independent of the 2,4-D concentration. Fig.3a shows that in Lp1 the percentage of explants that regenerated only green shoots decreased from 86% to 33% with increased 2,4-D concentrations. Simultaneously, the percentage of explants with albino shoots increased from 10% to 61% in the range 2,5 to 12,5 mg/l 2,4-D. Another diploid Lp variety gave similar results (data not shown). At 15 mg/l 2,4-D the total regeneration frequency was lowest (55%). Fig.3b shows that in Lm2 the effect of 2,4-D on shoot regeneration was less pronounced. The percentage of explants that formed only green shoots was lowest (14%) at 2,5 mg/l 2,4-D, while the frequency of explants with albino shoots was highest (57%) at this concentration. In the range from 2.5 to 15 mg/l 2,4-D there was a decrease in albino shoot regeneration from 58% to 33%.

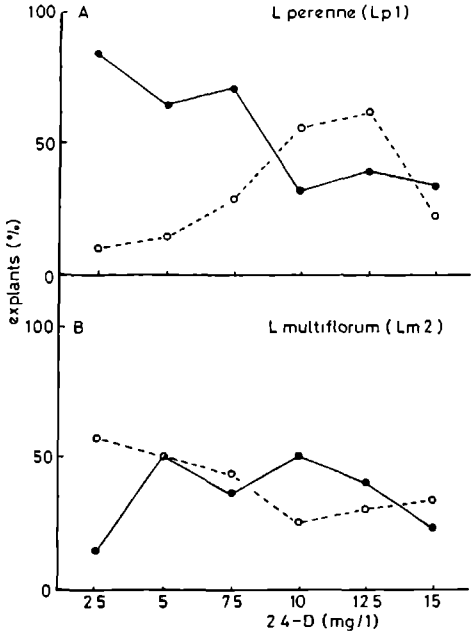

Fig. 3. The effect of different 2,4-D concentrations on the regeneration frequency of immature inflorescence-derived explants in one *L.perenne* and one *L.multiflorum* variety. The percentages are the means of 8 to 14 explants with compact callus; up to 9 pieces of compact callus were tested per explant.
●——● green shoots
○---○ albino shoots.

For both varieties, green shoots derived from cultures at all 2,4-D concentrations used, formed roots when subcultured to half strength MS0. Shoots regenerated from callus cultured at 12,5-15mg/l 2,4-D proliferated slowly and had a light green and vitrified appearance.

DISCUSSION

The immature inflorescence explants of the *Lolium perenne* and *Lolium multiflorum* varieties that were tested in this study, showed a high response (up to 100% of the explants) for compact callus induction and embryogenesis. Statistical analysis revealed a significant variety-growth conditions interaction for this response. For the induction of compact and embryogenic callus no optimal 2,4-D concentration in the culture medium could be determined. Regeneration of green plants from compact callus was readily achieved in all cultivars. However, even after only 3 to 4 months of culture albino shoots were also regenerating from all tested varieties. The frequency depended on the cultivar and increased with total culture age. Interestingly, explants from field-grown plants formed significantly more albino shoots than explants from greenhouse-grown plants. In the *L.perenne* variety tested, increasing concentrations of 2,4-D in the callus initiation medium resulted in a lower regeneration frequency of green shoots and a higher regeneration frequency of albino shoots.

Dale et al. (1981) reported that up to 40% of the explants from immature inflorescences of *L. multiflorum* formed compact callus. In that study 2-8mm long segments from inflorescences

up to 50mm long were cultured. By using only the two basal 2 mm segments of very young inflorescences (4-7mm long) we obtained a higher response for compact callus formation in *L. multiflorum* as well as in *L. perenne*. Embryogenic callus formation from immature maize embryos has been reported to be more dependent on the growth conditions of the donor plants than on the cultivar (Lu et al., 1983, Srinivasan and Vasil, 1986). Our observations show that in *Lolium* the developmental stage of the inflorescences apparently is an important factor for the formation of embryogenic callus.

Several studies report on the effect of 2,4-D on callus initiation and growth from explants of gramineous species (Conger et al., 1978,1982, Deambrogio and Dale, 1980, Fladung and Hesselbach, 1986, Lu and Vasil, 1982, Thomas and Scott, 1985). In most studies an optimal 2,4-D concentration for callus initiation and growth was determined, which varied with the species. Our results (table 2) show that for the induction of compact and embryogenic callus in the tested *Lolium* varieties no optimal 2,4-D concentration could be determined and cultures tolerated up to 15mg/l 2,4-D.

In *L.perenne* we observed that higher 2,4-D concentrations resulted in the regeneration of more albino shoots and less green shoots. In barley increasing 2,4-D levels in the callus initiation medium also resulted in a gradual decrease of green shoot regeneration (Deambrogio and Dale, 1980). There is evidence that prolonged in vitro culture in the presence of 2,4-D induces chromosome breakage in Nicotiana spp (Ronchi et al., 1976). In the present study callus cultured at increasing 2,4-D levels proliferated faster. The higher mitotic activity of these tissues might be accompanied with a higher mutation frequency, thus resulting in the regeneration of more albino plantlets. Of interest in this respect are observations made in our laboratory on callus cultures from *Poa pratensis* (Kentucky bluegrass). Here albino shoots were produced almost exclusively from friable, rapidly growing callus after prolonged culture periods (Van der Valk et al., 1989). There is little information regarding the effect of donor plant environment on plant regeneration. Plants grown in the field, as compared to plants grown in the greenhouse, are exposed to high climatic fluctuations such as light intensity, temperature, drought and wind. Physiological stress might block or mutate functional genes in the donor tissue. Subsequently, the alterations in the genome might be expressed in the emergence of albino shoots regenerating from these tissues. Our observations somewhat resemble those of Wang et al. (1978), who reported that anthers of rice are sensitive to temperature-induced albinism at the stage before the first mitosis of the mother cells.

The emergence of albino shoots from somatic tissue cultures in gramineous species has been reported earlier for *Lolium* (Dale, 1980, Dale et al., 1981, Dale and Dalton, 1983) and also for *Bromus* (Gamborg et al., 1970), *Hordeum* (Thomas and Scott, 1985), *Pennisetum* (Chandler and Vasil, 1984) and 5 temperate forage

grasses (Lo et al., 1980). Most workers found that only a minor proportion of young callus cultures formed albino shoots, but that the frequency rapidly increased with culture age (Chandler and Vasil, 1984, Dale et al., 1981, Lo et al., 1980). However, in anther culture albinism occurs more frequently. Efforts to understand and control albinism have been, therefore, mainly made by researchers working with anther culture in gramineous species (Clapham, 1973, Day and Ellis, 1984,1985, Sunderland and Huang, 1985, Wang et al., 1978). The true molecular basis of albinism is not yet known. The available information suggests that both nuclear and chloroplast genes might be involved in establishing albinism. Of interest is, in this respect, the work done by Day and Ellis (1984, 1985), who demonstrated the presence of large deletions in the chloroplast DNA of wheat and barley albino plantlets.

If morphogenic cell suspension cultures are to be initiated from explant-derived callus cultures it will be important to control the regeneration process from the beginning of culture. From this work with *Lolium* it is concluded that it is important to select varieties with a high regeneration frequency. To keep the percentage of albino shoots low the donor plants should be grown under conditions that avoid physiological stress of the plant tissue. These conditions can be better controlled in the greenhouse than in the field. To prevent loss of regeneration capacity the total culture period should be kept short. In order to obtain a high ratio of green to albino shoots it is, at least for *L.perenne*, favourable to use a culture medium containing a low level of 2,4-D.

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REFERENCES

Bühring J, Neubert K (1977) Results regarding the accelerated production of complete generations of *Lolium perenne* L. and *Dactylis glomerata* L. under artificial climate, in: E. WO-jah and H. Thons (Eds.), Proceedings XIII Int. Grassland Congress, Vol. 1, Akademie Verlag, Berlin, pp 407-409

Chandler SF, Vasil IK (1984) Optimization of plant regeneration from long term embryogenic callus cultures of *Pennisetum purpureum* Schum. (Napier grass). J Plant Physiol 117:147-156

Clapham D (1973) Haploid *Hordeum* plants from anthers in vitro. Z Pflanzenzüchtg 69:142-155

Conger BV, Carabia JV, Lowe KW (1978) Comparison of 2,4-D and 2,4,5-T on callus induction and growth in three Gramineae species. Environ Exp Bot 18:163-168

Conger BV, Hilenski LL, Lowe KW, Carabia JV (1982) Influence of different auxins at varying concentrations on callus induction and growth from embryo and leaf-tip explants in Gramineae. Environ Exp Bot 22(1):39-48

Dale PJ (1980) Embryoids from cultured immature embryos of *Lolium multiflorum*. Z Pflanzenphysiol 100:73-77

Dale PJ, Thomas E, Brettell RIS, Wernicke W (1981) Embryogenesis from cultured immature inflorescences and nodes of *Lolium multiflorum*. Plant Cell Tissue Organ Culture 1:47-55

Dale PJ, Dalton SJ (1983) Immature inflorescence culture in *Lolium*, *Festuca*, *Phleum* and *Dactylis*. Z Pflanzenphysiol 111:39-45

Dalton SJ (1988) Plant regeneration from cell suspension protoplasts of *Festuca arundinacea* Schreb., *Lolium perenne* L. and *L. multiflorum* Lam. Plant Cell Tissue Organ Culture, 12:137-140

Day A, Ellis THN (1984) Chloroplast DNA deletions associated with wheat plants regenerated from pollen: Possible basis for maternal inheritance of chloroplasts. Cell 39:359-368

Day A, Ellis THN (1985) Deleted forms of plastid DNA in albino plants from cereal anther culture. Current Genetics 9:671-678

Deambrogio E, Dale PJ (1980) Effect of 2,4-D on the frequency of regenerated plants in barley and on genetic variability between them. Cereal Research Communications 8(2):417-422

Fladung M, Hesselbach J (1986) Callus induction and plant regeneration in *Panicum bisulcatum* and *Panicum milioides*. Plant Cell Reports 3:169-173

Gamborg OL, Constabel F, Miller RA (1970) Embryogenesis and production of albino plants from cell cultures of *Bromus inermis*. Planta 95:355-358

Jones MGK, Dale PJ (1982) Reproducible regeneration of callus from suspension culture protoplasts of the grass *Lolium multiflorum*. Z Pflanzenphysiol 105:267-274

Lo PF, Chen CH, Ross JG (1980) Vegetative propagation of temperate forage grasses through callus culture. Crop Science 20:363-367

Lu C, Vasil IK (1982) Somatic embryogenesis and plant regeneration in tissue cultures of *Panicum maximum* Jacq. Amer J Bot 69(1):77-81

Lu C, Vasil V, Vasil IK (1983) Improved efficiency of somatic embryogenesis and plant regeneration in tissue cultures of maize (*Zea mays* L.). Theor Appl Genet 66:285-289

McCullagh P, Nelder JA (1983) Generalized Linear Models, Chapman & Hall, London pp

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473-497

Ozias-Akins P, Vasil IK (1982) Plant regeneration from cultured immature embryos and inflorescences of *Triticum aestivum* L. (wheat): Evidence for somatic embryogenesis. Protoplasma 110:95-105

Ronchi VN, Martini G, Buiatti M (1976) Genotype-hormone

interaction in the induction of chromosome aberrations: effect of 2,4-dichlorophenoxyacetic acid (2,4-D) and kinetin on tissue cultures from *Nicotiana* spp. Mutation Research 36:67-72

Santos MA, Torne JM (1986) A comparative analysis between totipotency and growth environment conditions of the donor plants in tissue culture of *Zea mays* L. J Plant Physiol 123:299-305

Srinivasan C, Vasil IK (1986) Plant regeneration from protoplasts of sugarcane (*Saccharum officinarum* L.). J Plant Physiol 126:41-48

Sunderland N, Huang B (1985) Barley anther culture-The switch of programme and albinism. Heriditas Suppl 3:27-40

Thomas MR, Scott KJ (1985) Plant regeneration by somatic embryogenesis from callus initiated from immature embryos and immature inflorescences of *Hordeum vulgare*. J Plant Physiol 121:159-169

Thompson JA, Abdullah R, Cocking EC (1986) Protoplast culture of rice (*Oryza sativa*) using media solidified with agarose. Plant Science 47:123-133

Torello WA, Mancino L, Troll J (1983) Initiation, growth, and maintenance of callus tissue derived from mature caryopses of perennial ryegrass (*Lolium perenne* L.). Rasen Turf Gazon 1:4-7

Torello WA, Symington AG (1984) Regeneration from perennial ryegrass callus tissue. Hort Sci 19(1):56-57

Van der Valk P, Zaal MACM, Creemers-Molenaar J (1989) Callus induction and plant regeneration from immature inflorescences and mature seeds of *Poa pratensis* L. (Kentucky bluegrass). Plant Cell Rep 7:644-647

Wang D, Vasil IK (1982) Somatic embryogenesis and plant regeneration from inflorescence segments of *Pennisetum purpureum* Schum. (napier or elephant grass). Plant Science Letters 25:147-154

Wang C, Sun C, Chu C, Wu S (1978) Studies on the albino pollen plantlets in rice, in: Proceedings of Symposium on Plant Tissue Culture, Pittman Publishing, Peking, pp 149-160

Yamada Y, Zhi-Qi Y, Ding-Tai T (1986) Plant regeneration from protoplast-derived callus of rice (*Oryza sativa* L.). Plant Cell Reports 5:585-88

CHAPTER 3

PLANT REGENERATION FROM SUSPENSION CULTURES AND PROTOPLASTS OF *LOLIUM PERENNE* L.

With: P. van der Valk, J.P.M. Loeffen and M.A.C.M. Zaal

Plant Science (1989) 63: 167-176

SUMMARY

Suspension cultures of 6 *Lolium perenne* varieties were initiated from immature inflorescence-derived compact callus or from seed-derived embryos. Established suspension cultures were used for the isolation of protoplasts. Protoplasts were cultured in liquid as well as in agarose-solidified medium.

Finely dispersed embryogenic suspension cultures with a capacity to regenerate green plants were obtained from 5 *L. perenne* varieties. Relatively more suspension cultures became established when immature inflorescence-derived callus was used for initiation. With few exceptions, the capacity to regenerate green shoots decreased with suspension culture age and was totally lost on average 25 weeks after suspension culture initiation.

Fast-growing, non-morphogenic suspension cultures yielded large numbers of protoplasts that formed numerous microcalli in liquid and agarose-solidified media. Protoplasts that were isolated from young, morphogenic suspension cultures with moderate growth rates formed no or few microcalli. However, one suspension culture which retained the capacity for plant regeneration for more than 17 months yielded protoplasts which formed microcalli. From these microcalli green shoots have been obtained in four experiments. Protoplast-derived plants have been established in soil.

INTRODUCTION

Cereals and grasses are recalcitrant to genetic manipulation for several reasons. *Agrobacterium tumefaciens* mediated gene transfer has been unsuccessful, because monocotyledons appear to be less susceptible to infection with *Agrobacterium* than dicotyledons (Vasil, 1988). Techniques such as somatic hybridization and direct DNA transfer can be applied, but require the use of protoplasts. However, plant regeneration from protoplasts of monocotyledonous species is restricted to a small number of species, while the regeneration frequency is often low. Protoplast-derived plants, that survived after transfer to soil, were obtained from *Oryza sativa* (Abdullah et al., 1986, Toriyama et al., 1986, Yamada et al., 1986, Kyozuka et al., 1987), *Saccharum officinale* (Srinivasan and Vasil, 1986, Chen et al., 1988), *Zea mays* (Rhodes et al., 1988), *Festuca arundinacea* (Dalton, 1988AB), *Lolium multiflorum* (Dalton, 1988A) and *Dactylis glomerata* (Horn et al., 1988).

In the above-mentioned studies protoplasts were isolated from fast growing, finely dispersed morphogenic suspension cultures. Under optimal culture conditions the morphogenic potential of protoplasts and the suspension cultures from which

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Abbreviations: 2,4-D: 2,4-dichlorophenoxyacetic acid, FW: fresh weight.

they were isolated was reported to be the same. The suspension cultures were initiated from explant-derived morphogenic callus, or directly from seed-derived mature embryos (Dalton, 1988AB). However, as can be concluded from the work reported by Maddock (1987) for wheat and by Lührs and Lörz (1988) for barley, it often appears to be difficult to obtain such fast growing, homogeneous and morphogenic suspension cultures. Moreover, conditions that ensure the successful establishment of such suspension cultures have not been determined or described in detail for most gramineous species.

In this paper is described the establishment of morphogenic suspension cultures of *Lolium perenne* and the isolation, culture and plant regeneration of suspension cell-derived protoplasts. We report the regeneration of plants from protoplasts of *L. perenne*, which were subsequently established in soil.

MATERIALS AND METHODS

Initiation and maintenance of suspension cultures

Suspension cultures of different *L. perenne* varieties were initiated directly from seed-derived embryos or from embryogenic callus cultures. According to the procedure reported by Dalton (1988A), mature seeds were sterilised in 10% hypochlorite, rinsed and soaked for 2 days in sterile tap water and sterilised for a second time. After rinsing thoroughly, mature embryos were dissected from 40 seeds, chopped and transferred to 5 ml MS10 medium (Murashige and Skoog basal salts supplemented with 10 mg/l 2,4-D, 3% sucrose at pH 5.8) in 60 ml plastic specimen flasks (Thovadex). Embryogenic callus was initiated from immature inflorescence segments of greenhouse-grown plants as described previously (Creemers-Molenaar et al., 1988). About 4-8 weeks after callus initiation, compact embryogenic callus was excised from explants of 2 to 5 plants (i.e. genotypes). The calli were mixed, chopped with a scalpel and 0.1 g FW was transferred to 5 ml MS10 medium in specimen flasks. Following initiation, cultures of both origins were treated identically. The cultures were incubated on a rotary shaker (140 rpm, 15mm amplitude) in continuous indirect light (200-400 lux) at 25°C. After 10 days the medium was replaced with MS5 medium (5 mg/l 2,4-D). Once a week, 1 to 2 ml of fresh medium was added up to a total volume of 12 to 15 ml. After 6-8 weeks suspension cultures growing well were transferred to 125 ml Erlenmeyer flasks and maintained by the weekly subculture of small pieces of compact callus into 25 ml fresh MS5 medium. Suspension cultures that proliferated well, but initially failed to form small callus pieces, were maintained by splitting up the total amount of callus between 2 flasks containing fresh medium every week.

Regeneration from suspension cultures

The regeneration capacity of the suspension cultures was tested by plating 0.5-1.0 g FW suspension on solid MS0 medium

(0 mg/l 2,4-D, 0.8% agar). The dishes were incubated in the dark for 1 week, followed by 1 week incubation in continuous indirect light (500 lux). For shoot regeneration, 12 pieces of compact, embryogenic callus (5 x 5 mm) were selected from each plate and subcultured on the same medium. These cultures were incubated in continuous direct light (7000 lux) for 3 to 4 weeks and finally the numbers of green and albino shoots were determined.

Protoplast isolation

For the isolation of protoplasts 1-2 g FW of small groups of cells was pipetted selectively from a suspension culture, 4 to 5 days after subculture, and added to 10 ml enzyme mixture containing: 0.5% Cellulase Onozuka RS (Yakult Honsha Co, Ltd), 0.5% Driselase (Sigma), 0.05% Pectolyase Y-23 (Seishin Pharmaceutical Co, Ltd) and 5mM MES buffer in CPW medium (Frearson et al., 1973) with 15% mannitol and 1.48 g/l CaCl2 2H2O, at pH 5.6. The cells were incubated overnight with gentle agitation (35 rpm, 15 mm amplitude) at 25°C in the dark. The protoplast suspension was passed successively through 55 and 20 µm nylon sieves and washed three times in CPW medium containing 13% mannitol with centrifugation steps of 3 minutes at 50 x *g*. The protoplasts were resuspended in RY-2 medium (Yamada et al., 1986) and the yield was determined using a haemocytometer. To determine the viability of the protoplasts and the presence of cell wall material 2 drops of the protoplast suspension were mixed with 1 drop of a solution containing 100 mg/l fluorescein diacetate (Janssen chimica) and 1 g/l calcofluor white (fluorescent brightener 28, Sigma) in RY-2 medium.

Protoplast culture and regeneration

The protoplasts were cultured at a density of 3.5×10^5 in 1 ml aliquots in plastic Petri dishes (Greiner, 35 mm, TC Quality) in liquid or 1% agarose-solidified (Seaplaque) RY-2 medium. The osmolality of the medium was adjusted to 920 mOs/kg with glucose. The cultures were incubated in the dark at 25°C. The plating efficiency was expressed as the percentage of plated protoplasts that had formed cell colonies (0.05-0.1 mm) after four weeks. This was determined by counting a representative sample of cell colonies per plate, using an inverted microscope. After 6 weeks, microcalli growing in liquid RY-2 medium (1 ml) were mixed with 2 ml N6 medium (Chu et al., 1975) without hormones, supplemented with 3% sucrose and 0.75% agarose. Microcalli growing initially in agarose-solidified RY-2 medium were subcultured by transferring agarose blocks to N6 medium without hormones, supplemented with 3% sucrose and 0.5% agarose. Calli were subcultured 1 to 2 times at 3 to 4 week intervals onto the same medium and were maintained in the dark. For plant regeneration compact and embryogenic calli were subcultured on the same medium and incubated in direct light (2000 lux, with a 16h/8h light/dark period) for 4-6 weeks. Shoots were then transferred to tubes containing MS0 medium as described before (Creemers-Molenaar et al., 1988).

Culture media

All media were filter sterilised, excluding the agar and agarose which were autoclaved as concentrated solutions at 121°C for 15 min.

RESULTS

Suspension culture initiation

Suspension cultures were initiated from mature embryos of 6 *L. perenne* varieties and from immature inflorescence-derived compact callus of 4 *L. perenne* varieties. Table 1 shows that established suspension cultures were obtained from 5 of the 6 varieties tested. Depending on the cultivar, 0-38% of the suspension cultures initiated from mature embryos and 20-100% of the suspension cultures initiated from compact callus became established. Young suspension cultures (<20 wks) were heterogeneous (Fig. 1A) and were composed of: 1) small groups (0.3 mm) of densely cytoplasmic cells 2) larger aggregates of compact callus 3) a minor fraction of watery, friable callus and 4) root primordia. Somatic proembryos, freely suspended in the medium or attached to the surface of compact callus, were often observed in these suspension cultures (Fig. 1B). Older suspension cultures tended to become less compact ,more friable and non-embryogenic. Several phenomena were observed that prevented suspension cultures from becoming established: 1) extensive root formation 2) browning of the callus 3) the production of watery, instead of compact callus 4) slow growth or 5) no disintegration of callus. Table 1 summarises the responses of the 6 varieties. Suspension cultures derived from the same cultivar often differed considerably with respect to their response in liquid medium. However, in Lp9 predominantly the formation of root primordia and roots prevented the routine establishment of suspension cultures.

Suspension culture regeneration

After plating on MS0, suspension culture callus continued to grow vigorously. Globular compact callus proliferated from smooth compact callus, as well as from watery callus. Two to three weeks after plating, somatic embryos were formed on the surface of compact callus. Young suspension cultures (10-15 weeks) often regenerated shoots within 2 weeks after plating, even before the transfer of compact callus to fresh MS0 plates (Fig. 1C). When pieces of compact or embryogenic callus were transferred to fresh MS0 plates, green or albino shoots regenerated directly from the callus or by the germination of somatic embryos. Up to 200 green shoots could be regenerated from 1 g FW suspension cells. Green shoots derived from regenerating suspension cultures of 5 *L. perenne* varieties were subcultured as described before (Creemers-Molenaar et al., 1988) and established in soil. Non-regenerating suspension cultures produced only watery callus that occasionally formed roots or compact callus without further differentiation.

Fig. 2 shows that the regeneration response varied between suspension cultures initiated from the same cultivar. Suspension culture Lp6T regenerated 165 green shoots per plate 9 weeks after initiation and gradually lost its regeneration capacity over a period of 34 weeks. Albino shoots regenerated only at low frequency from suspension culture Lp6T with little fluctuation. It was characteristic for suspension culture Lp6T that the callus 1) never dissociated into small callus pieces 2) was dark yellow in colour and 3) became highly embryogenic upon plating.

Compared with suspension culture Lp6T the regeneration frequencies of the suspension cultures Lp6B and Lp6D were considerably lower: only 35 and 20 green shoots per plate respectively regenerated nine weeks after suspension culture initiation. The competence for plant regeneration of cultures Lp6B and Lp6D was also lost earlier, 19-21 weeks after suspension culture initiation. In Lp6B the regeneration of albino shoots increased with suspension culture age, but decreased to zero rapidly 16 weeks after initiation. Loss of the capacity to regenerate green shoots was often observed to occur a few weeks earlier than that for albino shoots.

Although upon plating, the suspension callus of cultures Lp6B and Lp6D was highly embryogenic up to 14 weeks after suspension culture initiation, 16-17 week old cultures produced only non-embryogenic callus.

With few exceptions, established suspension cultures from the other varieties showed regeneration patterns similar to Lp6B and Lp6D and the capacity to regenerate green shoots was lost within 25 weeks after suspension culture initiation. This response occurred irrespective of the suspension culture origin.

Suspension culture Lp9A represents a remarkable exception to the general pattern of culture development. This suspension culture was initiated from immature inflorescence-derived callus and only became established 20 weeks after initiation. During the first 20 weeks this suspension culture was characterized by slow growth of yellow compact callus and root primordia with only a minor fraction of small callus pieces: removal of the small callus pieces at this stage resulted in growth retardation and browning of both fractions. After 20-25 weeks the growth rate of the suspension culture gradually increased and when small callus pieces were transferred to fresh medium the suspension culture continued to grow and

Table 1. Suspension culture initiation from mature embryos (e) and immature inflorescence-derived embryogenic callus (c) of different *L. perenne* varieties.

Variety	donor tissue	No. suspension cultures									
		initiated	established	regenerating shoots			showing response noted				
				G	A	NT	R	B	SG	FW	ND
Lp1	e	9	3	1	1	1	3	1	1	1	0
Lp1	c	2	2	2	2	0	/	/	/	/	/
Lp6	e	43	15	12	14	0	10	15	16	2	4
Lp6	c	1	1	1	1	0	/	/	/	/	/
Lp9	e	5	1	1	1	0	3	1	0	0	1
LP9	c	5	1	1	1	0	4	0	0	0	0
Lp12	e	20	2	0	0	0	5	9	9	7	5
Lp18	e	4	0	/	/	/	2	2	1	1	2
Lp18	c	2	2	1	1	1	/	/	/	/	/
Lp92	e	5	0	/	/	/	1	0	0	0	4

NT=not tested, /=not applicable, G=green, A=albino, R=roots and root primordia, B=browning of callus, SG=slow growth, FW=friable, watery, ND=no disintegration of callus.

became more homogeneous after each subculture. Fig. 2 shows that the regeneration response of suspension culture Lp9A was low and fluctuated between 0-7 green shoots per plate, with occasionally one albino shoot. However, this suspension culture has retained its regeneration capacity for more than 17 months. Pro-embryogenic structures were present in the suspension culture and embryogenic as well as watery friable callus was formed upon plating on solid medium. Shoots regenerated directly from the friable type of callus and occasionally from somatic embryos.

Protoplast isolation and culture

Protoplasts were obtained as a pure fraction without any notable debris (Fig. 3A) and 95-100% of the freshly isolated protoplasts were viable. In protoplast isolations from morphogenic suspension cultures a minor fraction of non-spherical, viable protoplasts was sometimes observed.

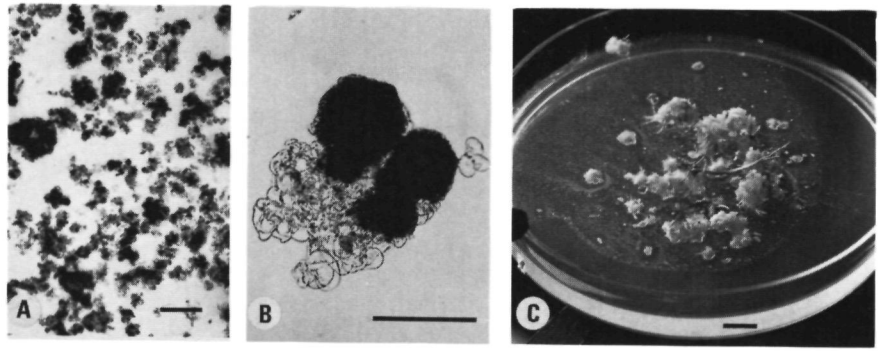

Fig. 1. Suspension culture of *Lolium perenne* (Lp6) initiated from seed-derived embryos. A) Heterogeneous suspension culture 12 weeks after initiation (bar= 1 mm). B) Somatic proembryos attached to watery callus (bar= 0.1 mm). C) Plant regeneration from a suspension culture 2 weeks after plating on solid MSO medium (bar= 1 cm).

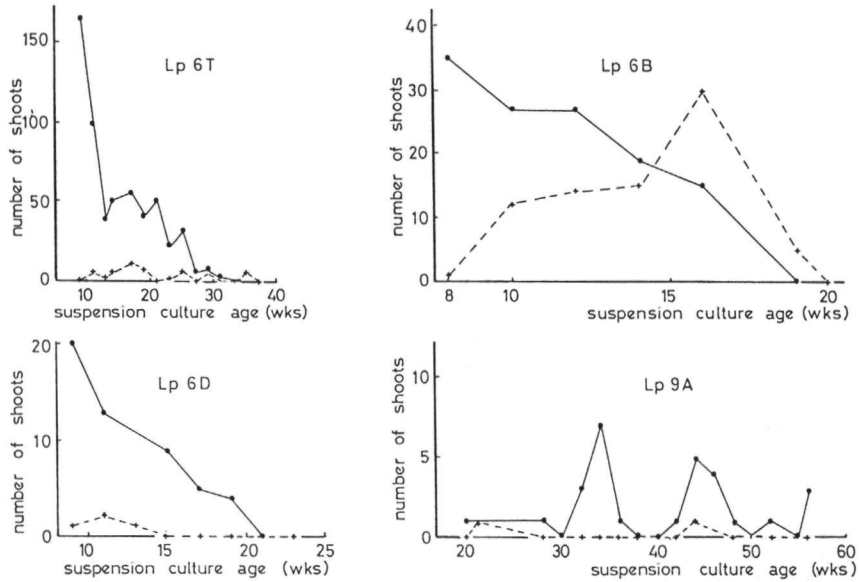

Fig. 2. Plant regeneration from suspension cultures of 2 varieties of *L. perenne* (Lp6 and Lp9), in relation to suspension culture age. Lp6 suspension cultures were initiated from mature embryos, Lp9 from immature inflorescence-derived callus. Three weeks after plating on MS0 12 pieces of compact callus (5 x 5 mm) were subcultured on the same medium. After 3-4 weeks the number of green and albino shoots was determined from two replicate plates.

+------+ albino shoots, ●————● green shoots.

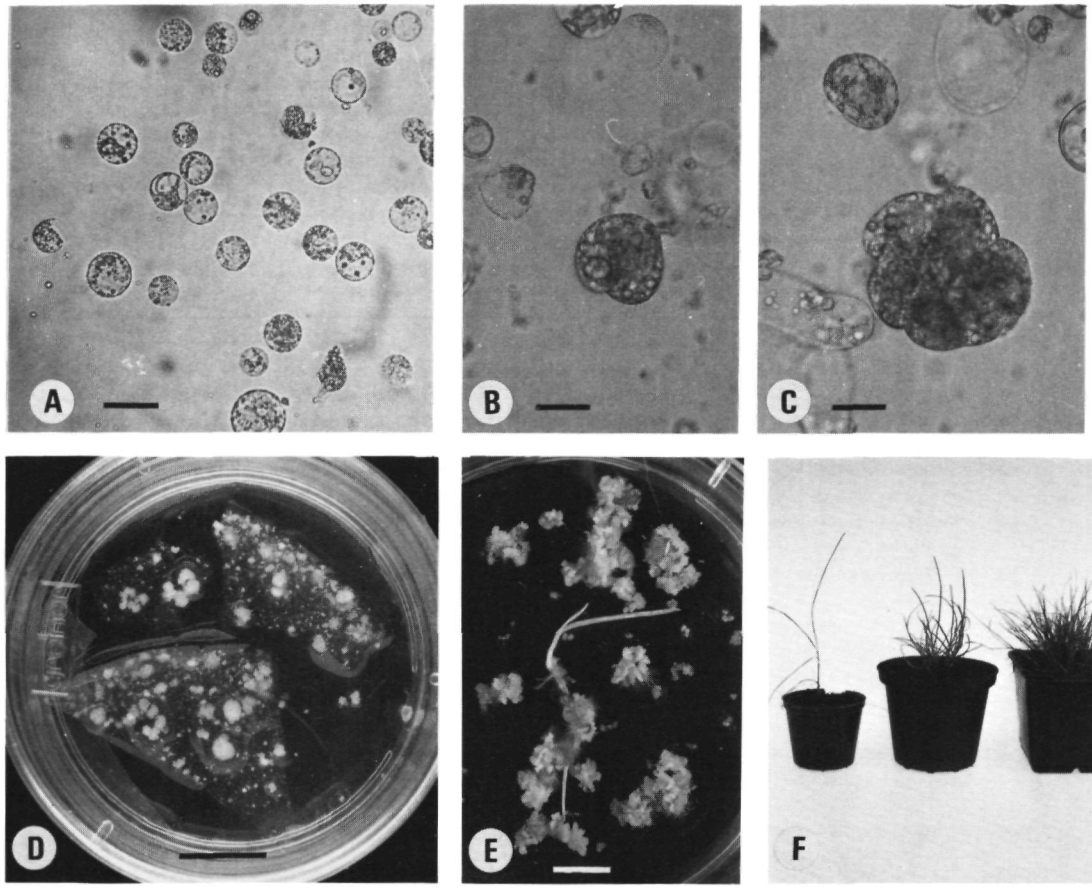

Fig. 3. Callus formation and plant regeneration from protoplasts of *Lolium perenne* (Lp9). A) Freshly isolated suspension culture protoplasts (bar= 20 um). B) First division of a protoplast in liquid RY-2 medium (bar= 20 ʌum). C) Protoplast-derived microcolony (bar= 20 ʌm). D) Microcalli in agarose-solidified RY-2 medium after subculture onto solid N6 medium (bar= 1 cm). E) Albino shoots from protoplast-derived microcalli (bar= 5 mm). F) Green plants regenerated from protoplasts.

Calcofluor white staining revealed that these protoplasts still retained cell wall material that covered part of the cell. The absence of a cell wall in the non-staining area was indicated by the ability of such cells to fuse in electrofusion experiments (data not shown). In protoplast isolations from the embryogenic suspension culture Lp6T, partly digested cells were always present. However, cell divisions were never observed and consequently microcalli were never obtained from such cells.

Yields of 2.0 X 10^6 protoplasts per gram FW suspension culture could be obtained from established suspension cultures 5-6 days after subculture. Table 2 shows that in Lp6, protoplast yield increased with suspension culture age. Young suspension cultures of Lp6 (9-10 weeks) released on average <1.0 x 10^5 protoplasts per gram FW suspension culture. Older suspension cultures (>20 weeks) yielded up to 1.4. x 10^7 protoplasts per gram FW. Protoplast yield from suspension culture Lp6T increased somewhat with suspension culture age, but remained low, irrespective of incubation time and the composition of the enzyme mixture.

After plating in liquid or agarose solidified medium, cell wall formation was observed after 3-4 days and first divisions started after 1 week (Fig. 3B). Cell colonies were microscopically visible 2-3 weeks after plating (Fig. 3C). Table 3 summarizes the plating efficiencies in liquid and agarose-solidified medium of 2 varieties. Compared with liquid medium, in agarose-solidified medium the plating efficiencies of protoplasts from the friable, watery suspension culture Lp6A was more than 2 times higher. For protoplasts from the more compact, morphogenic suspension culture Lp9A the plating efficiency in agarose was 5 times lower, compared with liquid medium. Generally, protoplasts from young regenerating suspension cultures formed no microcalli in agarose, but when the suspension cultures became more friable and less morphogenic the protoplasts responded better in agarose-solidified medium.

Protoplast regeneration

After the first subculture to solid N6 medium without 2,4-D, up to 5% (200-300) of the microcalli continued to grow (Fig. 3D), the other calli turned brown. After the second or third subculture of compact calli on the same medium 0-50% of the calli formed albino shoots (Fig. 3E). Albino shoots regenerated from protoplast-derived calli of 3 *L. perenne* varieties (Lp1, Lp6, Lp9). At a very low frequency (<1%) protoplast-derived calli of Lp6 and Lp12 regenerated light green shoots that bleached after 2-3 days and then turned brown. True green shoots were however obtained from 1-14% of the protoplast-derived calli of suspension culture Lp9A in 4 experiments: 20, 40, 43 and 66 weeks after suspension culture initiation (Table 2). In total, 7 green shoots developed into green plantlets of which 3 have sofar been established in soil

(Fig. 3F). These plants developed normally and needed no special treatment for maintenance. Flow cytometric analysis showed that these plants, like the donor plants, were diploid.

Table 2. Yield, plating efficiency and plant regeneration of protoplasts from suspension cultures of 3 *L. perenne* varieties (Lp6, Lp9 and Lp12). Protoplasts were cultured in liquid RY-2 medium. Suspension culture origin: e=mature embryos, c=immature inflorescence-derived callus. Shoots: a=albino, g=green.

cell line (origin)	culture age (weeks)	yield/ g FW x 10^6	plating efficiency (%)	culture response				
				callus	green spots	shoots a	shoots g	established plants
Lp6A (e)	19	0.4	0.2	+	-	+	-	-
	31	1.0	0.2	+	-	+	-	-
	33	14	0.2	+	-	+	-	-
Lp6T (e)	9	0.07	0	-	-	-	-	-
	23	0.2	0	-	-	-	-	-
	34	0.5	0	-	-	-	-	-
Lp6B (e)	14	0.1	0	-	-	-	-	-
	18	0.5	0	-	-	-	-	-
Lp6D (e)	10	0.1	0	-	-	-	-	-
	18	0.7	<0.01	+	+	-	-	-
	20	1.3	>1.0*	+	-	-	-	-
Lp9A (c)	20	0.2	<0.01	+	+	-	+	+
	28	3.7	0	-	-	-	-	-
	40	2.6	0.4	+	+	-	+	-
	43	1.0	0.1	+	-	+	+	-
	48	1.3	2.2	+	+	+	-	-
	66	1.4	1.3	+	+	+	+	-
Lp12A(e)	23	1.9	0.4	+	-	-	+	-
Lp12L(e)	10	0.11	0.5	+	-	-	-	-

*Strong aggregation of predominantly watery microcalli prevented exact calculation of the plating efficiency.

Table 3. Plating efficiency (%) of protoplasts from 2 *L. perenne* varieties (Lp6 and Lp9) in liquid and 1% agarose-solidified RY-2 medium. Plating efficiencies are presented ± SE values.

cell line	agarose	liquid
Lp6A[a]	0.5 ± 0.03	0.2 ± 0.03
Lp9A[b]	0.1 ± 0.07	0.5 ± 0.09

[a] The data are means of 3 experiments, performed within a period of 2 weeks.
[b] The data are means of 4 experiments, performed within a period of 7 weeks.
In every experiment plating efficiencies were determined from 2-3 replicate plates.

DISCUSSION

The results presented show that in *Lolium perenne* finely dispersed suspension cultures with a capacity for plant regeneration were established from immature inflorescence-derived morphogenic callus as well as directly from seed-derived embryos. Relatively more suspension cultures became established when immature inflorescence-derived callus was used for initiation. However, with few exceptions, the ability to regenerate green shoots was lost within 25 weeks after suspension culture initiation. Loss of regeneration capacity occurred from the moment that suspension cultures started to grow faster and was preceded by loss of the capacity of plated suspension culture callus to form somatic embryos.

Pronounced differences with respect to growth characteristics (Table 1) and regeneration (Fig. 2) were observed in cell lines derived from the same cultivar. Every suspension culture was initiated from a number of genotypes. It is not known whether one or more of the genotypes will, in time, dominate in proliferation. In that case the differences observed could be genotype-dependent. To exclude this source of variation it will be necessary to screen individual genotypes for their amenability to culture in liquid medium. This can be carried out by initiating suspension cultures with immature inflorescence-derived callus from individual genotypes.

The rapid loss of regeneration capacity was a striking and reproducible feature in most established suspensions cultures. These results are consistent with those reported by Dalton (1988B), who showed that suspension cultures of *Lolium* and *Festuca* retained a capacity to regenerate green plants up to 30 weeks after suspension culture initiation. Similar observations have been reported for embryo-derived suspension cultures from *Poa pratensis* (Van der Valk et al., 1988). The work presented showed that once a friable type of callus appeared in morphogenic

suspension cultures, this callus proliferated faster than the more compact organized callus, resulting in a non-morphogenic suspension culture. Remarkably, those suspension cultures that retained the ability to regenerate green shoots over prolonged periods, were characterized by moderate growth of mainly compact and pro-embryogenic cell groups.

In carrot callus cultures growth rate (to be interpreted as number of cell cycles) was demonstrated to be correlated with a decrease in embryogenic potential (Meyer-Teuter and Reinert., 1973). As reviewed by Bayliss (1980) and Vasil (1987) numerous data are available that relate the loss of regeneration capacity in cultured tissues to the occurrence of cytogenetically abnormal cells (aneuploidy, polyploidy). In wheat suspension cultures (*Triticum aestivum*) it was shown that selection for fast growing cells increased the proportion of cells with chromosome losses (Karp et al., 1987). Swedlund and Vasil (1985) presented data which showed that in *Pennisetum americanum* cytogenetically abnormal cells, already present in the explant material, divided in culture and proliferated faster than normal diploid cells.

It was shown here for *L. perenne* that plants regenerated from a more than one year old suspension culture with moderate growth rate were diploids, like the donor plants. Furthermore, the results presented demonstrate that a correlation exists between increased growth rate and loss of morphogenesis in *L. perenne* suspension cultures. However, cytological data of regenerated plants and suspension cells from suspension cultures with different growth rates and morphogenic capacities are necessary to determine whether a relation exists between cytological abnormalities and loss of morphogenesis.

The procedure followed here for the culture of *L. perenne* protoplasts differs from that reported by Dalton (1988AB) in the use of agarose, the addition of 0.8% foetal calfserum, lower concentrations of calcium and ammonium and exclusion of mannitol. Despite the different media compositions, the plating efficiencies in liquid medium are comparable. However, in agarose-solidified medium protoplasts formed microcalli in RY-2 medium, but not in PC-4 medium (Dalton, 1988AB).

Up until now only plantlets were recovered from protoplasts of *L. perenne* (Dalton, 1988AB). The work presented here shows that green plants that survived in soil have been regenerated from protoplasts isolated from a long-term regenerating suspension culture Lp9. These plants were phenotypically normal and cells contained the usual diploid chromosome number. From previous work with cultured immature inflorescences in *L. perenne* (Creemers-Molenaar et al., 1988) Lp9 was recognized as a highly responsive variety with respect to compact callus induction (up to 100% of the explants) and regeneration of green shoots (up to 96% of the compact calli). Our results suggest that for the initiation of suspension cultures it is important to select varieties with a high regeneration response from callus. The regeneration frequency from protoplasts is still low and limited to one cell line. For successful application of

protoplast-dependent genetic manipulation techniques the described procedure is an important step forward, but nevertheless requires further improvement.

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REFERENCES

Abdullah R, Cocking EC, Thompson JA (1986) Efficient plant regeneration from rice protoplasts through somatic embryogenesis. Bio/Technology 4:1087-1090

Bayliss MW (1980) Chromosomal variation in plant tissues in culture. Int Rev Cytol Suppl 11A:113-144

Chen WH, Davey MR, Power JB, Cocking EC (1988) Sugarcane protoplasts: factors affecting division and plant regeneration. Plant Cell Rep 7:344-347

Chu CC, Wang CC, Sun CS, Hsu C, Yin KC, Chu CY, Bi FY (1975) Establishment of an efficient medium for anther culture of rice through comparative experiments on the nitrogen sources. Sci Sinica 18:659-668

Creemers-Molenaar J, Loeffen JPM, Van der Valk P (1988) The effect of 2,4-Dichlorophenoxyacetic acid and donor plant environment on plant regeneration from immature inflorescence-derived callus of *Lolium perenne* L. and *Lolium multiflorum* L. Plant Sci 57:165-172

Dalton SJ (1988A) Plant regeneration from cell suspension protoplasts of *Festuca arundinacea* Schreb. (tall fescue) and *Lolium perenne* L. (perennial ryegrass). J Plant Physiol 132:170-175

Dalton SJ (1988B) Plant regeneration from cell suspension protoplasts of *Festuca arundinacea* Schreb., *Lolium perenne* L. and *L. multiflorum* Lam. Plant Cell Tissue Organ Culture 12:137-140

Frearson EM, Power JB, Cocking EC (1973) The isolation, culture and regeneration of Petunia leaf protoplasts. Dev Biol 33:130-137

Horn ME, Conger BV, Harms CT (1988) Plant regeneration from protoplasts of embryogenic suspension cultures of orchardgrass (*Dactylis glomerata* L.). Plant Cell Rep 7:371-374

Karp A, Wu QS, Steele SH, Jones MGK (1987) Chromosome variation in dividing protoplasts and cell suspensions of wheat. Theor Appl Genet 74:140-146

Kyozuka J, Hayashi Y, Shimamoto K (1987) High frequency plant regeneration from rice protoplasts by novel nurse culture methods. Mol Gen Genet 206:408-413

Lührs R, Lörz H (1988) Initiation of morphogenic cell-suspension

and protoplast cultures of barley (*Hordeum vulgare* L.). Planta 175:71-81

Maddock SE (1987) Suspension and protoplast culture of hexaploid wheat *Triticum aestivum* L.). Plant Cell Rep 6:23-26

Meyer-Teuter H, Reinert J (1973) Correlation between rate of cell division and loss of embryogenesis in longterm tissue cultures. Protoplasma 78:273-283

Rhodes CA, Lowe KS, Ruby KL (1988) Plant regeneration from protoplasts isolated from embryogenic maize cell cultures. Bio/Technology 6:56-60

Srinivasan C, Vasil IK (1986) Plant regeneration from protoplasts of sugarcane (*Saccharum officinarum* L.). J Plant Physiol 126:41-48

Swedlund B, Vasil IK (1985) Cytogenetic characterization of embryogenic callus and regenerated plants of *Pennisetum americanum* (L.) K. Schum. Theor Appl Genet 69:575-581

Toriyama K, Hinata K Sasaki T (1986) Haploid and diploid plant regeneration from protoplasts of anther callus in rice. Theor Appl Genet 73:16-19

Van der Valk P, Zaal MACM, Creemers-Molenaar J (1988) Regeneration of albino plantlets from suspension culture derived protoplasts of *Poa pratensis* L. (Kentucky bluegrass). Euphytica Sup pp 169-176

Vasil IK (1987) Developing cell and tissue culture systems for the improvement of cereal and grass crops. J Plant Physiol 128:193-218

Vasil IK (1988) Progress in the regeneration and the genetic manipulation of cereal crops. Bio/Technology 6:397-402

Yamada Y, Zhi-Qi Y, Ding-Tai T (1986) Plant regeneration from protoplast-derived callus of rice (*Oryza sativa* L.). Plant Cell Rep 5:85-88

CHAPTER 4

THE EFFECT OF GENOTYPE, COLD STORAGE AND PLOIDY LEVEL ON THE MORPHOGENIC RESPONSE OF PERENNIAL RYEGRASS (*LOLIUM PERENNE* L.) SUSPENSION CULTURES

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Plant Science (submitted)

SUMMARY

The effects of genotype, cold storage and ploidy level were investigated on suspension cultures of perennial ryegrass (*Lolium perenne* L.) in order to improve their morphogenic potential. For the establishment of cell suspension cultures, 28 plants that had been regenerated from protoplasts or suspension cultures, were analysed.

One protoplast-derived plant and five suspension-derived plants yielded calli from immature inflorescence explants which were amenable for suspension culture initiation. None of the callus cultures initiated from meristems of protoplast-derived plantlets proved suitable for suspension culture initiation. Cold storage for six weeks extended the regeneration competence period of the two perennial ryegrass suspension cultures examined, and even enhanced the regeneration frequency of one of these cultures.

The ploidy level of cells decreased from euploidy to aneuploidy with suspension culture age in nine of the suspension cultures analysed. For six of these cultures the decrease of ploidy level was associated with a reduction of the capacity for plant regeneration. Seventeen of 21 green shoots regenerated from two already sub-diploid suspension cultures were nevertheless diploid, three were octoploid and one was a mixoploid. Eighteen albino shoots were obtained from the same cultures. Of these, only four were diploid, whereas the remaining shoots were tetraploid, mixoploid or chimeras.

It was concluded that reduction of the morphogenic response in suspension cultures of perennial ryegrass was correlated with a decrease of the ploidy level. Furthermore, the establishment and the morphogenic response could be improved by cold storage and genotype selection respectively.

INTRODUCTION

The general approach to obtain plant regeneration from protoplasts in cereals and grasses has been the establishment of fast growing, morphogenic suspension cultures from which the protoplasts can then be isolated (Vasil, 1987). To date, such regeneration-competent suspension cultures have been obtained in most monocotyledonous species that are of economic importance, e.g. rice, wheat, maize, sugarcane, perennial ryegrass, italian ryegrass, orchard grass and tall fescue. Subsequently, plant regeneration from suspension-derived protoplasts has also been achieved in these species (reviewed in Creemers-Molenaar and Beerepoot, 1991). However, rapid loss of the morphogenic capacity of established suspension cultures occurs frequently and imposes serious limitations to the routine application of e.g.

Abbreviations: FW= fresh weight, Lp= Lolium perenne, MS= Murashige and Skoog

protoplast-dependent genetic manipulation techniques (Dalton, 1988, Van der Valk et al., 1988, Creemers-Molenaar et al., 1989). Most perennial ryegrass suspension cultures lose their morphogenic potential within 6-12 months after initiation (Creemers-Molenaar et al., 1989). Decrease of the capacity for plant regeneration may be the result of "in vitro" culture-induced chromosomal variation. A high degree of chromosome instability was correlated with a loss of the morphogenic capacity in tissue cultures of tobacco (Murashige and Nakano, 1967, Zagorska et al., 1974). Several reports describe the occurrence of chromosomal variation in cultured plant tissues (Ogura, 1990, Pijnacker and Sree Ramulu, 1990). Chromosomal variation, e.g. polyploidy and aneuploidy, has been shown to increase with culture age in embryogenic callus of pearl millet (Swedlund and Vasil, 1985) and several dicotyledonous species (Ogura, 1990).

In addition to the ploidy level of cultures, both the origin of the explants used for callus induction as well as pretreatment of the cultures may influence the regeneration response. To maintain the competence for plant regeneration in cultured plant tissues, cold storage has been shown to be adequate in *Lotus corniculatus* (Orshinsky and Tomes, 1985) and sour cherry (Borkowska, 1990). An increase in the frequency of somatic embryogenesis was observed in *Medicago truncatula*, when regenerated plants were used as an explant source (Nolan et al., 1989).

With the aim to improve the morphogenic potential of suspension cultures of perennial ryegrass (*Lolium perenne* L.) to be used in genetic manipulation experiments, investigations were carried out on the effect of genotype, cold storage and ploidy level. Cell suspension cultures were initiated from regenerated plants in order to select responsive genotypes. Data are reported on the regeneration frequency of suspension cultures after cold storage and on flow cytometric determination of nuclear DNA contents in suspension cultures and regenerated plants.

MATERIALS AND METHODS

Suspension culture initiation, maintenance and plant regeneration

Diploid (2n=2x=14) perennial ryegrass cultivars and breeding lines were used. The suspension cultures studied were cultures of Lp22 (cultivar Aurora), Lp25 (breeding line Jon 401) and Lp9 (breeding line B200). These cultures were either initiated from immature inflorescence-derived callus, or directly from mature embryos, as has previously been described (Creemers-Molenaar et al., 1989). The procedure for callus induction from explants of immature inflorescences was also applied to induce callus from meristem explants of protoplast-derived plantlets. The cultures were maintained under standard conditions by weekly subculture of 5 g FW (fresh weight) suspension callus in 25 ml MS5 medium (containing 5 mg/l 2,4-D) and were incubated on a rotary shaker

(110 rpm) at 25°C in the dark. Suspension cultures were considered to be "established" when the callus had become finely dispersed and adequate numbers of protoplasts could be isolated from the cultures. For determination of the morphogenic capacity, 0.5-1 g FW suspension callus was plated onto solid MS0 regeneration medium and subcultured as described previously (Creemers-Molenaar et al., 1989). The regeneration frequency was determined as the number of green and albino shoots per g FW suspension callus.

Cold storage

The effect of cold storage on the plant regeneration frequency was investigated using Lp25M1, a finely dispersed, 18 wk old, mature embryo-derived, suspension culture and Lp22mix, a more heterogeneous 13 wk old, mature embryo-derived, suspension culture. Taking each in turn, callus from a single batch was divided between 4 Erlenmeyer flasks and to each flask 25 ml fresh MS5 medium was added. From both suspension cultures two Erlenmeyer flasks were maintained continuously under standard conditions and two were stored at 4°C without shaking. After 6 weeks, the cold-stored cultures were returned to standard conditions. Samples from cultures kept under standard conditions, as well as from cold-stored cultures, were taken at 2-weekly intervals during the periods before; during and after storage and were plated onto 3 dishes of solid regeneration medium. The suspension cultures were maintained as long as they were competent for plant regeneration.

Genotype selection

Callus was induced on explants from immature inflorescences of field-grown plants which had been regenerated from protoplasts or suspension cultures in the previous year as described in Creemers-Molenaar et al. (1988). The protoplast-derived plants (p) originated from suspension culture lines Lp22K (plants 22Kp1, 22Kp7 and 22Kp20) and Lp22M (plants 22Mp6 and 22Mp7). The suspension-derived plants (s) originated from suspension culture lines Lp22M (plant 22Ms4) and Lp9T (plants 9Ts1, 10, 37, 40 and 46). In addition, callus was induced from meristem explants of 17 protoplast-derived plantlets. These plantlets originated from 3 separate protoplast isolations from suspension culture Lp22KK (plantlets 22KKp 1-6, 7-12 and 13-17). Initially, the callus cultures were subcultured completely. Then, four weeks after the second subculture, a semi-compact, morphogenic callus type was selected and 1 g was transferred to 15 ml MS5 suspension culture medium. Further conditions were similar to those described for suspension culture initiation, maintenance and plant regeneration. The cultures were scored for their ability to become established (callus type) and for plant regeneration frequency.

Flow cytometric determination of ploidy level

Six suspension cultures (Lp22KK 1-6), initiated with meristem-

derived callus as described above, and 3 suspension cultures (I, III and V), initiated with mature embryos of Lp22, were examined for DNA content and plant regeneration frequency during the course of successive subcultures. The DNA content of samples from the suspension culture cells was determined at 2-4 weekly intervals by analyses of nuclear suspensions by flow cytometry as described previously (J. Creemers-Molenaar, submitted for publication). A nuclear suspension, prepared from leaves of *Nicotiana plumbaginifolia* plantlets, was used as an internal standard for all the samples. Leaf nuclei from greenhouse-grown diploid and tetraploid *Lolium perenne* varieties were used to determine 2c and 4c values. Simultaneously, samples of the suspension cultures were plated onto 2 dishes of MS0 medium to determine the regeneration frequency. Callus-derived and mature embryo-derived suspension cultures were examined from weeks 0-20 and 8-20 respectively. In addition, the DNA contents of green and albino shoots, regenerated from 18-20 wk old suspension cultures of Lp22K1 and Lp22V, were determined.

RESULTS

Cold storage

The perennial ryegrass suspension culture Lp25M1 lost its competence for plant regeneration 22 wks after suspension culture initiation, when it had been maintained under standard conditions (Fig. 1a). However, when it was stored at 4^{0}C between wks 18-24 the regeneration frequency (number of shoots per gram fresh weight) did not decrease during this period. Instead, the regeneration frequency of this culture appeared to increase after cold storage and this elevated level was maintained for more than four weeks after its return to standard conditions. This cold-stored suspension culture only lost its capacity for plant regeneration 34 wks after suspension culture initiation.

Similar results were obtained after cold storage of suspension culture Lp22mix (Fig. 1b). For this culture, the regeneration frequency during cold storage and under standard conditions were comparable. However, after transfer of the cold-stored culture to standard conditions the competence for plant regeneration was always higher and was maintained 4 wks longer as compared to the culture that had been kept continuously under standard conditions.

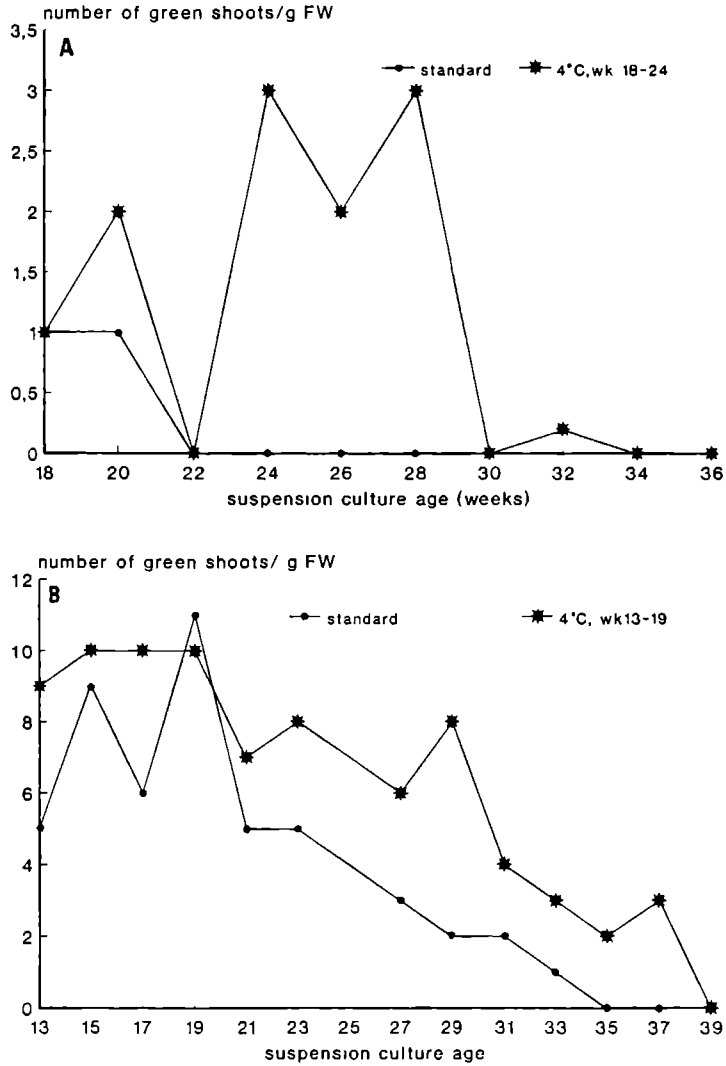


Fig. 1. The effect of cold storage on the plant regeneration frequency of mature embryo-derived suspension cultures of *Lolium perenne* L. (a) Suspension culture Lp25M1; storage at 4°C from wk18-24, (b) Suspension culture Lp22mix; storage at 4°C from wk 13-19. Standard= maintenance under standard (25°C) conditions (see Materials and Methods). All values represent the average of 6 dishes (2 Erlenmeyers/treatment)culture.

Table 1. Tissue culture response of suspension cultures initiated using meristem-derived and immature inflorescence-derived calli of selected genotypes of *Lolium perenne L.*

Genotype	Callus[a] source	Suspension callus type[b] 1	2	3	4	Regeneration period[c] (wks)
22KKp1	m	c	l	y	+	11
2	m	c	l	y	+	22
3	m	c	l	y	+	22
4	m	c	l	y	+	>22
5	m	c	l	y	+	>22
6	m	r	l	b	-	12
7	m	c	l	y	+	6
8	m	c	l	y	+	18
9	m	c	l	y	+	18
10	m	c	l	y	+	18
11	m	c	l	y	+	18
12	m	c	l	y	+	18
13	m	c	l	y	+	20
14	m	c	l	y	+	18
15	m	c	l	y	+	11
16	m	c	l	y	+	18
17	m	c	l	y	+	18
22Kp 1	ii	c	l	b	±	14
7	ii	c	l+s	y	+	>23
22Kp 20	ii	c	l	y	-	10
22Mp 6	ii	c	l	y	-	10
22Mp 12	ii	c	l	y	-	10
22Mc 4	ii	c	l+s	y	±	10
9Tc 1	ii	c	s	y	+	18
10	ii	c	s	y	+	15
37	ii	c	l+s	y	+	23
40	ii	c	s	y	++	<2
46	ii	c	l+s	y	+	22

The genotypes are as described in Materials and Methods.

[a] m= meristem-derived callus, ii= immature inflorescence-derived callus.

[b] **1: callus type**; c= compact, r= roots. **2: degree of friability** l= large aggregates (>1mm), s= small aggregates (<1mm). **3: colour**; y= yellow, b= brown. **4: growth rate**; -= slow, ±= moderate, += fast, ++= very fast.

[c] regeneration competent period, starting from suspension culture initiation.

Genotype selection

The results on suspension culture initiation using calli from selected genotypes of perennial ryegrass are shown in Table 1. None of the suspension cultures initiated using meristem-derived callus from the protoplast-derived plantlets 22KKp1-17 became finely dispersed. These cultures, except for 22KKp6, showed similar growth characteristics by proliferating a compact type of callus that did not release small aggregates. The duration of the regeneration-competent period for the majority of these cultures was 18 wks. One suspension culture which had been initiated from immature inflorescence-derived callus from plant 22Kp7 became finely dispersed and maintained a high capacity for plant regeneration for more than 23 wks (27 green shoots/g FW at wk 21). None of the suspension cultures initiated with immature inflorescence-derived callus of 22M plants became established. These cultures lost their capacity for plant regeneration within 10 wks from suspension culture initiation. In contrast, the initiation of suspension cultures using immature inflorescence-derived callus of 9T plants was successful, without exception. Furthermore, it was observed that suspension cultures in which part of the callus remained as larger aggregates, lines 9Ts37, 9Ts46, retained their morphogenic capacity longer (22-23 wks) than cultures, lines 9Ts1, 9Ts10, 9Ts40, which became finely dispersed (18, 15, <2 wks).

Ploidy level

The relation between suspension culture age, the regeneration frequency and the ploidy level of suspension callus of 3 callus-derived and 3 mature embryo-derived suspension cultures is shown in Fig. 2. During the period when the suspension cultures were examined, only suspension culture Lp22I became established.

Lp22KKp5. At the time of suspension culture initiation, meristem-derived callus of plantlet 22KKp5 appeared to be already tetraploid (2n=4x) (Fig.2a). The ploidy of the protoplast-derived plantlet was tetraploid also (results not shown). The ploidy of the suspension culture initiated from the tetraploid callus gradually decreased from 2n=3.9x to 2n=3.7x between weeks 0-16 and had decreased further to 2n=2.3 by wk 19. During this period the regeneration frequency decreased from 43 to 5 green shoots/g FW. An increase of the regeneration frequency of albino shoots (24-109/g FW) was observed between wks 12-18.

Lp22KKp4. The suspension callus of culture Lp22KKp4 was diploid at the time of initiation (Fig. 2b). The ploidy of the suspension gradually decreased from 2n=2.0x to 2n=1.7x. The regeneration frequency of albino shoots was low during the entire culture period, whereas the regeneration frequency of green shoots was high and, although fluctuated, did not decrease with time. Similar patterns were observed with respect to the ploidy levels and regeneration frequencies of green shoots for suspension lines Lp22KK2 and Lp22KKp3 (results not shown).

Lp22KKp6. The ploidy of suspension culture Lp22KKp6 decreased from 2n=1.9x-2n=1.4x between wk0 and wk16 (Fig. 2c). The initial

capacity for the regeneration of green shoots was low (0-4 shoots/g FW) and was lost within 14 wks after suspension culture initiation. The regeneration frequency of albino shoots first decreased (40-4 shoots/g FW) between weeks 2 and 6 and then increased (4-40 shoots/g FW) between weeks 14-18.

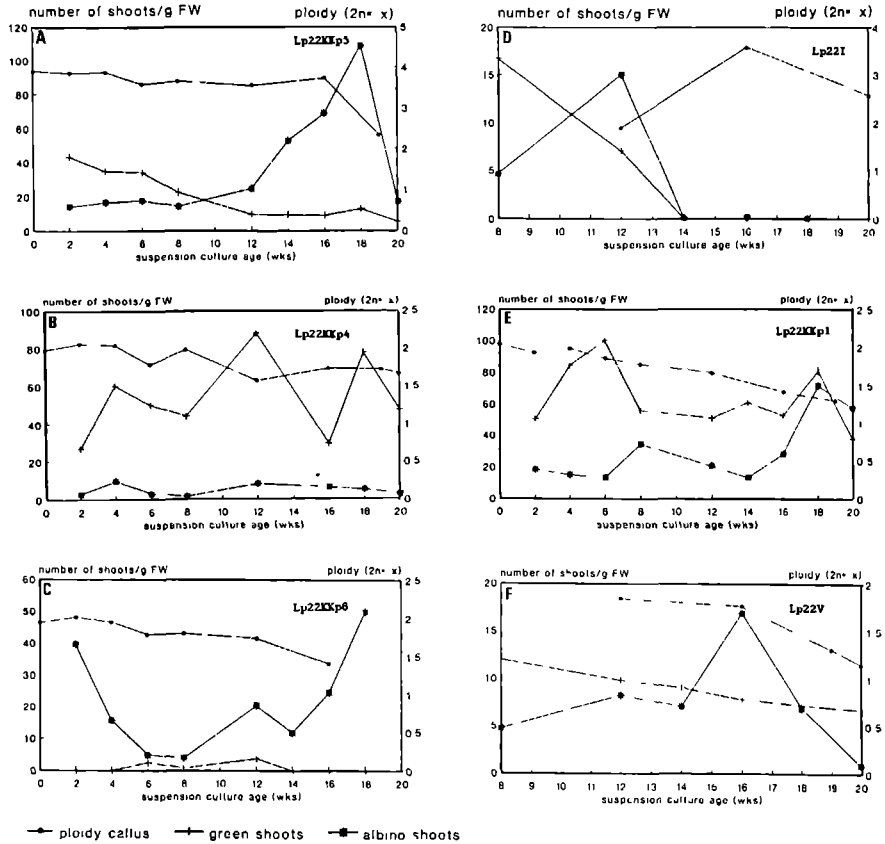
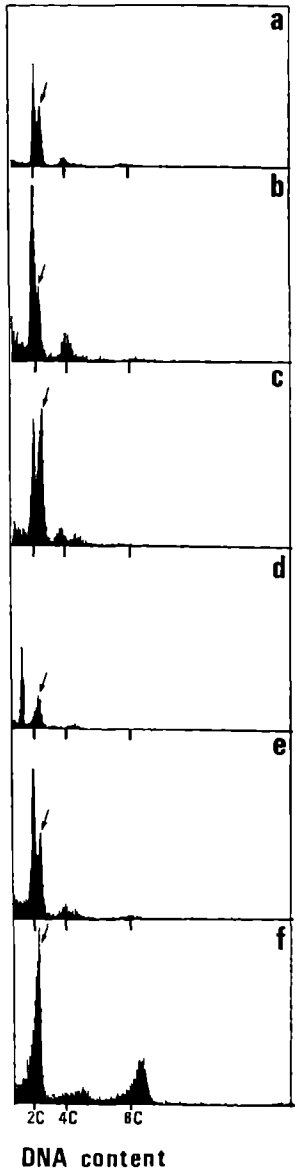


Fig. 2. Ploidy levels, as determined by flow cytometry, and plant regeneration frequencies of 6 suspension cultures of *Lolium perenne* L. in relation to suspension culture age. See Materials and Methods for origin of the suspension cultures: (a) Lp22KKp5; (b) Lp22KKp4; (c) Lp22KK6; (d) Lp22I; (e) Lp22KKpI; (f) Lp22V.



Lp22I. As shown in Fig. 2d, the mature embryo-derived suspension culture Lp22I was still diploid at wk 12 (2n=1.9). However, by wk 16 the callus had become almost tetraploid (2n=3.6). During this period the culture became finely dispersed with a fast growth rate. The capacity for plant regeneration rapidly decreased from 17 shoots/g FW (wk8) to 0 shoots/g FW (wk14). From wk 16 the ploidy of the suspension callus started to decrease again from 2n=3.8 to 2n=2.6.

Lp22KKp1. The ploidy of this suspension had gradually decreased from 2n=2.0x to 2n=1.3x by week 20 (Fig. 2e). However, the regeneration frequency of green shoots remained high over the entire culture period, only to decrease (81-37 green shoots/g FW) between weeks 18 and 20.

Lp22III, Lp22V. The development of the ploidy levels and the regeneration frequencies of the mature embryo-derived cultures Lp22V (Fig. 2f) and Lp22III (results not shown) were similar to that of suspension culture Lp22KK5. In all three cultures the decrease of the DNA content was highest between wk 16 and wk 20. During this period the regeneration frequency of albino shoots increased, whereas the regeneration frequency of green shoots continued to decrease.

Fig. 3. Frequency distributions of the relative nuclear DNA content of *Lolium perenne* L. cells: (a) seed-derived plant of cultivar 22; (b-d) mature embryo-derived suspension culture Lp22V after 12, 16, 19 wks; (e, f) green shoots regenerated from the 18 wk old suspension culture Lp22V. The arrows point to the 2c(2x) peak of the *Nicotiana plumbaginifolia* internal standard, which was mixed with each *L. perenne* sample.

The ploidy levels of green and albino shoots regenerated from suspension culture Lp22k1 in wk 20 and culture Lp22V in wks 19 and 20 are given in Table 2. Of the 21 green regenerants analysed, 17 were diploid, 3 were octoploid and 1 was a mixoploid with diploid and tetraploid cells. While the octoploid shoots were all derived from a single callus, all the other shoots examined originated from different calli. Of the 18 albino shoots analysed, only 4 were diploid, 4 were tetraploid, 6 were mixoploid (diploid + tetraploid) and 4 were chimeras with diploid and aneuploid cells with intermediate DNA values between triploid (3x) and pentaploid (5x). No sub-diploid shoots were observed.

To give an example of the results described above, the DNA histograms of seedlings, suspension callus and regenerated shoots from suspension line Lp22V are shown in Fig. 3. The seed-derived plant was diploid (2n=2x, Fig. 3a), the 12 wk, 16 wk and 19 wk old suspension calluses had ploidy levels of 2n=1.85x (Fig. 3b), 2n=1.75x (Fig. 3c) and 2n=1.3x (Fig. 3d) respectively. Of the three green shoots regenerated from an 18 wk old suspension culture, two were diploid (Fig. 3e) and one was octoploid (Fig. 3f).

Table 2. Ploidy levels of regenerated green and albino shoots from perennial ryegrass suspension cultures Lp22KK1 and Lp22V.

Susp line	Susp age (wks)	Shoots[a]	Ploidy level					
			<2x	2x	4x	2x+4x	8x	Chim[b]
22KK1	20	g (11)	0	10	0	1	0	0
		a (17)	0	4	4	6	0	3
22V	18	g (8)	0	5	0	0	3	0
		a (1)	0	0	0	0	0	1
22V	20	g (2)	0	2	0	0	0	0

The suspension cultures were initiated as described in Materials and Methods. The suspension cultures were plated onto regeneration medium at a stage when the ploidy level of suspension cells had decreased from 2x to 1.3 to 1.15x.

[a] = albino, g= green, the number of shoots in parentheses.

[b] The four albino shoots had a chimeric composition: 2.5x and 3.8x, 2x and 4.4x, 2x and 4.9x and 2x and 3x.

DISCUSSION

Experiments designed to select for genotypes with a high response for callus induction and suspension culture initiation, yielded one suitable protoplast regenerant and five suitable suspension culture regenerants. Established suspension cultures could only be successfully obtained from callus derived from immature inflorescences of field-grown plants. The observation that none of the meristem-derived callus cultures were amenable for suspension culture initiation may indicate that meristems are unsuitable explant types for this purpose. To date, the use of meristem-derived callus for suspension culture initiation in perennial ryegrass has not been reported. However, these results may also reflect a genotype- specific response, in that the protoplast-derived plantlets from which the meristems were excised might have originated from a single, non-responding genotype. The uniform response of plants regenerated from the established suspension culture Lp9T suggests that the ability of immature inflorescence-derived callus to produce finely dispersed and morphogenically-competent suspension cultures is a trait that is genotype-dependent.

Extension of the period of regeneration-competence of perennial ryegrass suspension cultures was achieved by low temperature storage of the cultures at 4°C in the dark. For one culture, cold storage for 6 weeks even enhanced the regeneration frequency. However, our results do not permit an explanation of the effects of cold storage.

With the aim to improve the regeneration competence period and to find causes for loss of the regeneration capacity in suspension cultures of perennial ryegrass, we have estimated the degree of ploidy level by analysing the DNA content of suspension cells during successive subcultures. For nine of the suspension cultures examined, a gradual decrease of the ploidy levels of 15-42% was observed during the initial culture period of 20 weeks. Decline of the ploidy level occurred in diploid as well as in tetraploid suspension cultures. In 6 suspension cultures the competence for green plant regeneration decreased simultaneously with a decrease of the ploidy level of 25-42% in the suspension cells. Nevertheless, the majority of green shoots which had regenerated from two sub-diploid suspension cultures (2n=1.15-1.3x) were normal diploids, whereas a few were octoploid. These results suggest that for the cell lines examined, only diploid or polyploid cells were capable of regenerating green plants. Complete loss of the regeneration capacity for green shoots might therefore only occur when all regeneration-competent suspension cells have been lost through dilution by potentially faster-growing sub-diploid or sub-polyploid cells or have eventually become sub-diploid or sub-polyploid. The observation made in this study that, for three out of the nine suspension cultures examined, a 15-25% decrease of the ploidy level did not affect the competence for plant regeneration might suggest that in these cell lines a portion of diploid, regeneration-competent cells was

stably maintained.

The present results that chromosome elimination often occurs in cell suspension cultures, is in agreement with the previous reports in *Triticum aestivum* (Karp et al., 1987, Kao et al., 1970), *Solanum tuberosum* (Pijnacker et al., 1986) and *Glycine max* (Kao et al., 1970). However, in these plant species, no data were reported on the regeneration response of the suspension cultures. In tobacco cultures Murashige and Nakano (1967) and Zagorska et al.(1974) demonstrated that chromosomal instability was related to the loss of morphogenic capacity. As suggested by these authors, the cause of aneuploidy, which frequently occurred, might be the result of chromosome breakage, an event that has been shown to increase with tissue age (Ahloowalia, 1983). However, the present results are in contrast with those reported in the latter publication. Plants regenerated from a triploid callus culture, initiated from a triploid embryo of hybrid origin (diploid *L. multiflorum* X tetraploid *L. perenne*), showed wide variation with respect to chromosome numbers. Gradual loss of chromosomes was observed in plants regenerated from successive subcultures. The hybrid nature of this callus might explain the different results. The only perennial ryegrass suspension culture that became established (Lp22I) changed almost to tetraploid level during the initial culture period of 16 weeks. During its shift from sub-diploid to sub-tetraploid level, the culture started to grow faster, suggesting that the polyploid cells may have a selective advantage over diploid cells. In addition, during the period that the culture became tetraploid, the regeneration capacity rapidly declined to zero. An upward shift in the ploidy level with age of already-aneuploid cells has previously been reported for pith callus cultures of *N. tabacum* (Murashige and Nakano, 1967). As suggested by these authors for *Nicotiana* and by Halperin (1986) for other species, aneuploidy can cause disturbance of the gene balance, which can be further intensified by polyploidization. This disturbed gene balance might thus suppress the morphogenic response in cultured tissues. The rapid decline in the morphogenic capacity of the perennial ryegrass suspension culture, was probably caused by the combined effects of aneuploidization and polyploidization.

Further, in contrast to green shoots, it was observed that albino shoots that had been regenerated from two sub-diploid suspension cultures displayed a high incidence of polyploidy and aneuploidy. The capacity to regenerate albino shoots often increased when the regeneration of green shoots started to decrease (see also Creemers-Molenaar et al., 1989). These results suggest that the occurrence of albino shoots from perennial ryegrass suspension cultures is indicative of an intermediate step in the increase of chromosomal variation (from euploidy to both aneuploidy and polyploidy), which ultimately results in complete loss of the morphogenic capacity of the cells.

Mathias and Fukai (1986) have demonstrated in wheat that a specific chromosome is involved in the morphogenic response of

callus. In this regard, further investigations on suspension cultures of *L. perenne.*, differing in their degree of chromosome loss and competence for plant regeneration, might shed light on the localization of genes that determine the morphogenic response in tissue cultures of this species.

In the present study it has been found that the decline in the capacity for plant regeneration in perennial ryegrass suspension cultures is correlated with a decrease of the ploidy level. Pretreatment of suspension cultures by cold storage extended the regeneration competence period. Among plants that had previously been regenerated from established suspension cultures and protoplasts, genotypes could be selected that showed a good response for callus induction and, subsequently, suspension culture initiation.

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REFERENCES

Ahloowalia BS (1983) Spectrum of variation in somaclones of triploid ryegrass. Crop Science 23:1141-1147

Borkowska B (1990) Influence of low temperature storage on regenerative capacity of sour cherry cultures. Fruit Sci Rep 17(1):1-7

Creemers-Molenaar J, Loeffen JPM, van der Valk P (1988) The effect of 2,4-Dichlorophenoxyacetic acid and donor plant environment on plant regeneration from immature inflorescence-derived callus of *Lolium perenne* L. Plant Sci 57:165-172

Creemers-Molenaar J, van der Valk P, Loeffen JPM (1989) Plant regeneration from suspension cultures and protoplasts of *Lolium perenne* L. and *Lolium multiflorum* L. Plant Sci 63:167-176

Creemers-Molenaar J, Beerepoot LJ (1991) *In vitro* culture and micropropagation of ryegrasses (*Lolium* species), in: Biotechnology in Agriculture and Forestry vol. 19: "High-Tech and Micropropagation III, Y.P.S. Bajaj (Ed.). Springer Verlag, Heidelberg (in press).

Dalton SJ (1988) Plant regeneration from cell suspension protoplasts of *Festuca arundinacea* Schreb., *Lolium perenne* L., and *L. multiflorum*, Lam. Plant Cell Tissue Organ Culture 12:137-140

Halperin W (1986) Attainment and retention of morphogenic capacity *in vitro*. In: Cell culture and somatic cell genetics of plants, Vol. 3, Plant regeneration and genetic variability, I.K. Vasil (Ed.). Academic Press, Inc, Orlando, pp 3-47

Jackson JA, Dale PJ (1988) Callus induction, plant regeneration and an assessment of cytological variation in regenerated plants of *Lolium multiflorum* L. J Plant Physiol 132:351-355

Kao KN, Miller RA, Gamborg OL Harvey BL (1970) Variations in chromosome number and structure in plant cells grown in suspension cultures. Can J Genet Cytol 12:297-301

Karp A, Wu QS, Steele SH, Jones MGK (1987) Chromosome variation in dividing protoplasts and cell suspensions of wheat. Theor Appl Genet 74:140-146

Mathias RJ, Fukui K (1986) The effect of specific chromosome and cytoplasm substitutions on the tissue culture response of wheat (*Triticum aestivum*) callus. Theor Appl Genet 71:797-800

Murashige T, Nakano R (1967) Chromosome complement as determinant of the morphogenic potential of tobacco cells. Am J Bot 54:963-970

Nolan KE, Rose RJ, Gorst JR (1989) Regeneration of *Medicago truncatula* from tissue culture: increased somatic embryogenesis using explants from regenerated plants. Plant Cell Rep 8:278-281

Ogura H (1990) Chromosome variation in plant tissue culture. In: Bajaj Y.P.S.(ed.): Biotechnology in agriculture and forestry, vol. 11: Somaclonal variation in crop improvement I. Springer Verlag, Berlin, Heidelberg, pp 49-84

Orshinsky BR, Tomes DT (1985) Effect of long-term culture and low temperature incubation on plant regeneration from a callus line of birdsfoot trefoil (*Lotus corniculatis* L.). J Plant Physiol 119:389-397

Pijnacker LP, Hermelink JHM, Ferwerda MA (1986) Variability of DNA content and karyotype in cell cultures of an interdihaploid *Solanum tuberosum*. Plant Cell Rep 5:43-46

Pijnacker LP, Sree Ramulu K (1990) Somaclonal variation in potato: a karyotypic evaluation. Acta Bot Neerl 39(2):163-169

Swedlund B, Vasil IK (1985) Cytogenetic characterization of embryogenic callus and regenerated plants of *Pennisetum americanum* (L.) K. Schum. Theor Appl Genet 69:575-581

Van der Valk P, Zaal MACM, Creemers-Molenaar J (1988) Regeneration of albino plantlets from suspension culture derived protoplasts of *Poa pratensis* L. (Kentucky bluegrass). Euphytica Suppl:169-176

Vasil IK (1987) Developing cell and tissue culture systems for the improvement of cereal and grass crops. J Plant Physiol 128:193-218

Zagorska NA, Shamina BZ, Butenko RG (1974) The relationship of morphogenic potency of tobacco tissue culture and its cytogenetic features. Biol Plant 16:262-274

CHAPTER 5

OPTIMIZING THE CULTURE OF PERENNIAL RYEGRASS PROTOPLASTS BY CONDITIONED MEDIUM, O-ACETYLSALICYLIC ACID AND ANTIOXIDANTS

With: F. A. van Eeuwijk, and F. A. Krens

Journal of Plant Physiology (submitted)

SUMMARY

The effects of conditioned medium, O-acetylsalicylic acid (ASA) and antioxidants on the plating efficiency and plant regeneration of suspension-derived protoplasts have been investigated with the aim to improve the procedure for protoplast culture in perennial ryegrass. Conditioning of the protoplast culture medium was indispensable for the proliferation of microcalli from protoplasts derived from young, regeneration-competent suspension cultures and, in addition, enhanced microcallus formation from protoplasts from older suspension cultures. The addition of ASA to the protoplast culture medium improved the plating efficiency of protoplasts, but not the regeneration frequency. The antioxidants vitamin C, E and n-propyl gallate did not improve the culture of perennial ryegrass protoplasts. However, the addition of other antioxidants (superoxide dismutase, catalase, glutathione, glutathione peroxidase and phospholipase A2) involved in the reduction of peroxides, to the protoplast isolation medium and the culture medium increased the plating efficiency.

INTRODUCTION

Plant regeneration from protoplasts has been achieved in most monocotyledonous species tested to date. However, except for rice, it is still difficult to obtain reproducible high plant regeneration frequencies from protoplasts of the grasses and cereals. Protoplasts have been used in transformation and somatic hybridization experiments, with the aim to apply techniques for genetic engineering. Such experiments often include treatments, e.g. polyethylene glycol (PEG), electroporation or electrofusion, that have been shown to reduce the plating efficiency of protoplasts. To enhance the probability of obtaining transformed plants or somatic hybrid plants, the basic protoplast culture conditions should therefore be as optimal as possible.

One of the methods to improve the plating efficiency of protoplasts is the use of conditioned medium. Actively-dividing cells release cellular metabolites, often referred to as conditioning factors (CF's), into the culture medium, which can stimulate the growth of microcalli from cultured protoplasts (Schröder et al., 1989). After addition of medium in which suspension cells had previously been grown to the

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Abbreviations: ASA, O-acetylsalicylic acid; CAT, catalase; CF('s), conditioning factor(s); CM, conditioned medium; FW, fresh weight; GSH, glutathione; GSH-Px, glutathione peroxidase; MS, Murashige & Skoog; n-PG, n-propyl gallate; PE, plating efficiency; Plase A2, phospholipase A2; SOD, superoxide dismutase.

protoplast culture medium, plating efficiencies of suspension cell protoplasts were shown to be increased significantly for *Zea mays* (Somers et al., 1987) and *Dactylis* (Horn et al., 1988).

Another factor that involves the culture of protoplasts is the toxic effect of ethylene. ASA (O-acetylsalicylic acid) is known as a factor that inhibits ethylene release from cultured plant cells and has also been shown to affect the plating efficiency of protoplasts (Carlswell et al., 1989; Shillito et al., 1989).

In addition, under stress-inducing conditions, e.g. protoplast isolation, PEG-treatment or electrofusion oxidizing compounds, e.g. free radical species, are released that influence the viability of protoplasts (Ishii, 1987,1988). Free radicals reduce the stability of the protoplast membrane by a process of lipid peroxidation (Cutler et al., 1989). Furthermore, it was shown that the addition of natural or synthetic antioxidants to the protoplast isolation and culture medium significantly increased protoplast stability in maize (Saleem and Cutler, 1987), the frequency of colony formation from rice protoplasts (Ishii, 1988) and both the PE and plant regeneration frequency from protoplasts in sugar beet (Krens et al., 1990).

Radicals are formed in cell membranes under physiological stress, e.g. the hydroxyl radical $OH^{\cdot}$, which can initiate lipid peroxidation. These radicals are strong enough to abstract a hydrogen atom from a fatty acid (Halliwell and Gutteridge, 1989). Under aerobic conditions the thus-formed carbon radical reacts with molecular oxygen (O_2) to form a peroxy radical. Peroxy radicals are converted to lipid peroxides by abstracting hydrogen atoms from other lipid molecules, thus stimulating a chain reaction of lipid peroxidation.

The experiments described in this paper were carried out to improve the plating efficiency and the plant regeneration frequency from suspension-derived perennial ryegrass protoplasts. First an improved protoplast culture medium was developed by the addition of conditioned medium. The plating efficiency was then further enhanced by the addition of O-acetylsalicylic acid or antioxidants. The antioxidants tested were chosen because of their expected mode of activity, which could be either preventative or terminative (Halliwell and Gutteridge, 1989). Antioxidants of the first type, e.g. catalase, superoxide dismutase, glutathione, glutathione peroxidase and vitamin C, can prevent the formation of, or scavenge already-produced free radicals. Antioxidants of the second type, e.g. vitamin E, n-propyl gallate, glutathione, phospholipase A2 and glutathione peroxidase, can scavenge intermediate radicals formed during lipid peroxidation or repair oxidative damage in membranes. Five antioxidants, which were found to improve the plating efficiency when added in specific combinations, were tested further to determine optimal concentrations and to identify possible interactions between these antioxidants.

MATERIALS AND METHODS

Protoplast isolation and culture

Protoplasts were isolated from suspension cultures of *Lolium perenne* L. as described previously (Creemers-Molenaar et al., 1989). The suspension cultures were either initiated from immature inflorescence-derived callus (lines 9A, 9T and B8) or from seed-derived embryos (lines 22C, G and M, lines 6A, DD and TT) (Creemers-Molenaar et al., 1989).
For the isolation of protoplasts 1-2 g FW suspension callus was added to 10ml CPW enzyme mixture (Frearson et al.,1973), with or without antioxidants. The isolated protoplasts were cultured (Creemers-Molenaar et al., 1989) for 6 weeks in RY-2 medium (Yamada, 1986), or RY-2 medium supplemented with conditioned RY-2 medium (see next section), conditioned medium + ASA or conditioned RY-2 medium + antioxidants. After 6 weeks, microcalli were transferred onto solid MS medium, supplemented with 2.5 mg/l 2,4-D (MS2.5) without antioxidants or ASA. The plating efficiency (PE) was determined after 3-4 weeks and was expressed as the percentage of plated protoplasts that had formed cell colonies. The regeneration frequency was expressed as the number of green and albino shoots/3.5×10^5 originally plated protoplasts, visible after the second subculture on solid MS0 medium in the light.

Conditioned medium

Suspension culture 9A was subcultured every 7 days by transfering 5 g FW suspension cells to 25 ml of fresh MS5 medium. Four days after subculture the conditioned culture medium was collected, passed through a 20 μm mesh nylon sieve and stored at -20°C. After thawing, the conditioned medium was centrifuged for 5 min at 350 x *g* and the supernatant was filtered successively through a Whatman glass fibre filter and a cellulose ester membrane filter (Schleicher & and Schuell, ME25, 0.44 μm). Conditioned medium and RY-2 medium (Yamada et al.,1986) were mixed at a 1:1 ratio after which the pH was adjusted to 5.6 and the osmolality to 910-920 mOsm/kg with glucose. Conditioned RY-2 medium (RY-C) was filter sterilized (Millipore, Millex-GV, 0,22μm) and stored at -20°C in 10 ml aliquots.

O-acetylsalicylic acid (ASA)

A pH-adjusted, 0.5% (w/v) stock solution of O-acetylsalicylic acid (BDH) was prepared in Milli-Q water and added to RY-C medium to give final concentrations of 0-100 mM.

Antioxidants

Glutathione peroxidase (GSH-Px), phospholipase A2 (PLase A2) and superoxide dismutase (SOD) were purchased from Hoechst; n-propyl gallate (n-PG), glutathione (GSH) and catalase (CAT) from Sigma; DL-tocopherol (acid ester disodium salt of vitamin E) from ICN and L-ascorbic acid (vitamin C) from BDH. pH-adjusted stock solutions (10-30 x concentrated) of GSH, GSH Px, Plase A2 and SOD

were prepared in CPW medium or RY-C medium and added to the isolation or culture medium at concentrations as described in the "Results" section. CAT, n-PG and, vitamins E and C were dissolved directly in RY-C or CPW medium. After addition, the pH and the osmolality were adjusted to the appropriate values. Filter-sterilized media containing antioxidants were stored at -20°C and thawed directly before use.

Experimental design for the antioxidants SOD, CAT, GSH, GSH-Px and Plase A2

Previous results (Creemers-Molenaar and Van Oort, 1990), describing the positive effects of the antioxidants SOD, CAT, GSH, GSH-Px and Plase A2 on the PE of perennial ryegrass protoplasts, merited further investigation of the dependence of the PE on the concentrations and possible interactions between the antioxidants.

An appropriate statistical design was found to be plan 8A.6 from Cochran & Cox (1957). Based on previous results, optimal concentrations (coded 0) were chosen for each antioxidant, and these concentrations were used as the origin of the design (see Table 1). Further, the concentrations of the antioxidants were raised or lowered in fixed steps (coded -2, -1, 1, 2) in such a way that 33 concentration combinations were made (Table 2). These 33 combinations were distributed over two blocks, representing two days. The antioxidants SOD and CAT were added to the enzyme mixture, GSH, GSH-Px and Plase A2 were added to the protoplast culture medium. As a control treatment, protoplasts were also isolated and cultured without antioxidants.

Table 1. Concentrations of 5 antioxidants, used in a response surface experiment to determine optimal conditions for the PE and microcallus growth of suspension-derived perennial ryegrass protoplasts.

antioxidant	-2	-1	origin 0	1	2	step width
SOD (u/ml)	50	100	**150**	200	250	50
CAT (u/ml)	200	600	**1000**	1400	1800	400
GSH (mM/ml)	0.2	0.6	**1.0**	1.4	1.8	0.4
GSH-Px (u/ml)	2.50	3.75	**5.00**	6.25	7.50	0.75
PlaseA2 (u/ml)	55	65	**75**	85	95	10

Abbreviations: See Materials and Methods.

RESULTS

Conditioned medium

The effect of medium conditioning is shown in Table 3. Conditioning of the protoplast culture medium had a positive effect on the PE of protoplasts in all the cell lines tested. Protoplasts from young suspension cultures formed few (line 22C) or no (lines 6AT and 6DD) microcalli in RY-2 medium, while in conditioned medium (RY-C) the PE was up to 0.45%. The PE of protoplasts from a 2 year old suspension culture Lp9A (exp.1-6) increased reproducibly when the protoplasts were cultured in RY-C medium. Conditioned medium harvested from another suspension culture (line 6JJ) gave similar results compared to conditioned medium from suspension line 9A (exp.5-6). The PE of protoplasts cultured in RY-2 medium that had been mixed with freshly prepared suspension medium was the same (exp.5-6), and in one case higher (exp.3), as compared to RY-2 medium

Table 3. The effect of conditioned medium on the PE of suspension-derived perennial ryegrass protoplasts.

			protoplast culture medium[a]				
cell line	exp (nr)	suspension culture age (wks)	RY-2	RY-C[b]	RY-C[c]	CM[d]	RY-MS[e]
9A	1	100	1.1 (2)	1.6 (2)	/	/	/
9A	2	100	0.17(3)	0.35(3)	/	/	/
9A	3	100	0.01(4)	0.72(4)	/	0(4)	0.15(4)
9A	4	100	0.06(2)	0.17(2)	/	/	/
9A	5	100	0 (2)	0.2 (2)	0.2(2)	/	0
9A	6	100	0 (2)	0.2 (2)	0.2(2)	/	0
6AT	7	23	0 (3)	0.1 (3)	/	/	/
6DD	8	18	0 (2)	0.45(2)	/	/	/
22C	9	8	0.01(2)	0.03(2)	/	/	/
22C	10	9	0.01(2)	0.08(2)	/	/	/
22J	11	9	0.03(3)	0.3 (3)	/	/	/

Suspension culture line 9A is not regeneration competent, suspension culture lines 6AT, 6DD, 22C and 22J are competent for the regeneration of green shoots. Values within brackets are the number of dishes examined.

[a] Media as described in materials and methods section. [b] RY-2 medium conditioned with suspension medium from line 9A. [c] RY-2 medium conditioned with suspension medium from line 6JJ. [d] conditioned medium that has not been mixed with RY-2 medium and has been adjusted to the standard pH and osmolality. [e] RY-2 medium mixed with freshly prepared suspension medium.

alone. When only conditioned suspension medium was used protoplasts showed budding and no cell division was observed (exp.3). Conditioned medium harvested 4 days after subculture gave optimal results. The activity of conditioned medium was not affected by freezing and thawing (results not shown). Based on these results, RY-C medium, as described in Materials and Methods, was used in all further experiments.

O-acetylsalicylic acid

The addition of 10-20 mg/l ASA to the RY-C protoplast culture medium improved the PE of protoplasts 1-3 times (Fig. 1). However, at 100 mg/l ASA a negative effect on the PE was observed in all experiments. The increase in the number of microcalli formed in the presence of low concentrations of ASA has to date been reproduced for a total of 6 suspension culture lines. However, despite the increase of the number of calli that proliferated, the regeneration frequency of green shoots from protoplasts cultured in the presence of ASA was lower compared to protoplasts cultured without ASA (Table 4).

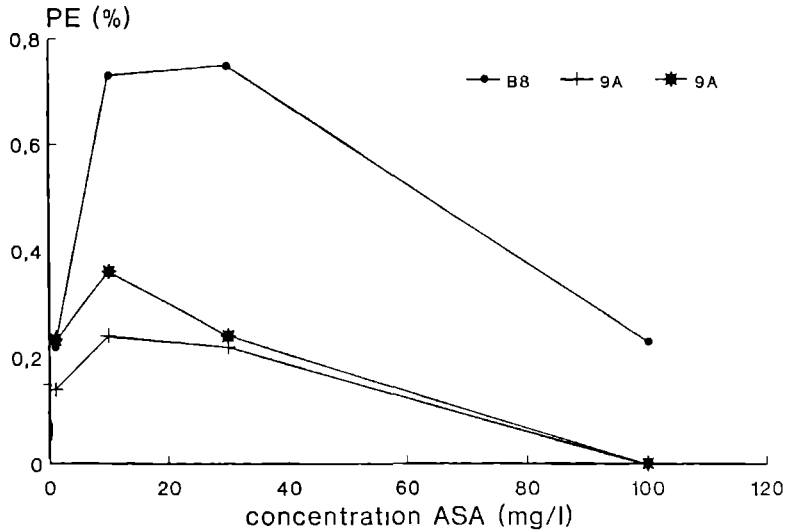

Fig. 1. The effect of the addition of ASA to the protoplast culture medium on the plating efficiency of suspension-derived protoplasts of *Lolium perenne* L., lines 9A and B8. PE was determined as the percentage of plated protoplasts that formed microcalli after 4 weeks of culture.

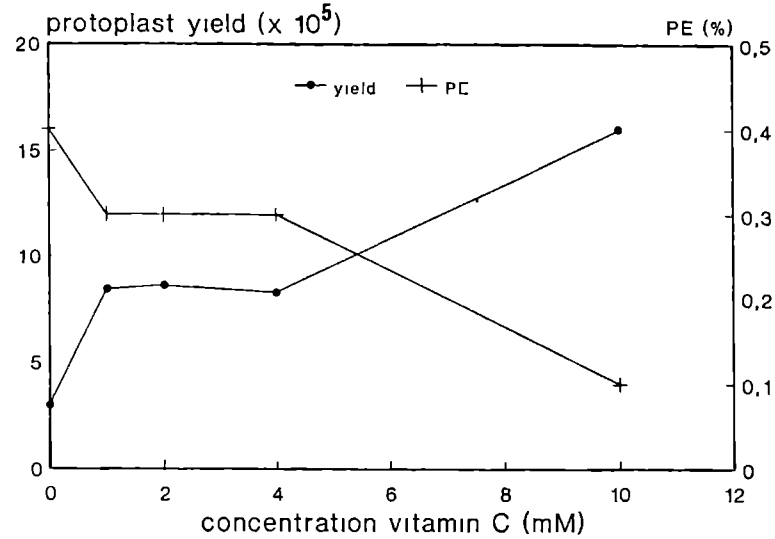

Figure 2. The effect of the addition of vitamin C to the protoplast isolation medium on the yield and the plating efficiency of suspension-derived protoplasts (line 22G) of *Lolium perenne* L. The yield was determined as the number of protoplasts isolated from 1 g FW suspension callus. PE was determined as the percentage of plated protoplasts that formed microcalli after 4 weeks of culture.

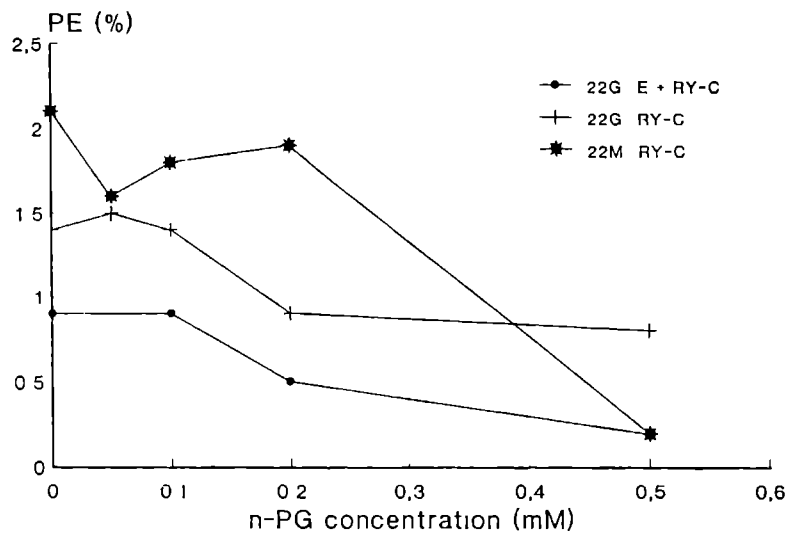

Figure 3. The effect of the addition of n-PG to the protoplast isolation medium (E) and/or the protoplast culture medium (RY-C) on the plating efficiency of suspension-derived protoplasts of *Lolium perenne* L., lines 22G and 22M. PE was determined as the percentage of plated protoplasts that formed microcalli after 4 weeks of culture.

Vitamins C and E

The results presented in Fig. 2 show that the presence of vitamin C in a range of 0-10 mM during protoplast isolation increased the protoplast yield from suspension culture 22G up to 5 times. However, the PE of protoplasts isolated under these conditions was decreased by up to 4 times. The negative effect of vitamin C on the PE of cultured protoplasts was observed, both when vitamin C was added to the enzyme mixture and to the culture medium and when it was added to the culture medium alone.

The addition of vitamin E to the enzyme mixture and/or the protoplast culture medium in the range 0.1-2.0 mM did not influence the protoplast yield or PE (results not shown). Neither was the PE of protoplasts improved by the simultaneous addition of vitamin C and E at a ratio of 10:1 (5 and 0.5 mM respectively).

n-PG

When n-PG was added to the protoplast isolation and/or culture medium in the range 0-0.5 mM, the PE's of protoplasts from suspension lines 22M and 22G were unaffected. The PE was decreased at the higher concentrations of n-PG (Fig. 3). Similar results were obtained with suspension culture line 9T.

SOD, CAT, GSH, GSH-PX and Plase A2

The observed PE's at the concentration combinations as prescribed by the experimental design of Cochran and Cox (1957) are given in Table 2. The observed PE at the origin of the second block was deemed to be aberrantly low and, as a consequence, was left out of further analyses. Analysis of the response surface subsequently revealed that the choice for the origin of the design represented conditions at which indeed an optimal PE was obtained. At this chosen point the concentrations of SOD, CAT, GSH, GSH-PX and Plase A2 were 150 u/ml, 1000 u/ml, 1.0 mM, 5.0 u/ml and 75 u/ml respectively. Compared to a control treatment where no antioxidants were added (PE= 0.2), the addition of antioxidants to the enzyme mixture alone (PE= 0.5), or to the enzyme mixture and the culture medium at the concentrations given above (PE= 1.0), increased the PE 2, 5 and 5 times respectively (Fig. 4). In addition, according to the fitted model, equally optimal conditions for the PE might be expected for all combinations where GSH and GSH-Px are kept at the origin, whereas SOD, CAT and Plase A2 vary away from the origin in the ratio 2;1;-1 respectively. As an example, some sets of expected optimal conditions are given in Table 5. The first column of Table 5 can be read as SOD having a distance of (50 -150)/50=-2 coded units from the origin, CAT (600-1000)/400=-1 unit and Plase A2 (85-75)/10=1 unit, while GSH and GSH-Px remain at the origin.

Further analysis of the response surface showed that changing the concentrations of certain antioxidants away from optimal conditions might lead to a rapid decrease of the PE. If the concentrations of antioxidants are changed according to the ratio SOD;CAT;GSH;GSH-Px;Plase A2=2;-1;-1;-1;2 the PE of cultured

protoplasts is expected to decrease rapidly. A similar but weaker response is expected for the ratios 0;2;-1;0;0 and 0;0;1;1;1.

Table 2. The effect of antioxidants on the PE of suspension-derived protoplasts of line 9A.

concentrations of antioxidants					PE	
SOD	CAT	GSH	GSH-Px	Plase A2		
						I*
-1	-1	1	-1	1	1.00	
1	-1	-1	-1	-1	0.41	
-1	1	-1	-1	-1	0.38	
1	-1	-1	-1	1	0.29	
-1	-1	1	-1	-1	0.47	
1	-1	1	-1	1	0.38	
-1	1	1	-1	1	0.51	
1	1	1	-1	-1	0.71	
-1	-1	-1	1	-1	0.65	
1	-1	-1	1	1	0.53	
-1	1	-1	1	1	0.54	
1	1	-1	1	-1	0.76	
-1	-1	1	1	1	0.56	
1	-1	1	1	-1	0.48	
-1	1	1	1	-1	0.23	
1	1	1	1	1	0.55	
0	0	0	0	0	0.86	
0	0	0	0	0	1.00	
0	0	0	0	0	0.99	
0	0	0	0	0	1.00	
0	0	0	0	0	1.10	
0	0	0	0	0	0.92	
						II*
-2	0	0	0	0	0.35	
2	0	0	0	0	0.35	
0	-2	0	0	0	0.18	
0	2	0	0	0	0.28	
0	0	-2	0	0	0.29	
0	0	2	0	0	0.29	
0	0	0	-2	0	0.37	
0	0	0	2	0	0.26	
0	0	0	0	-2	0.20	
0	0	0	0	2	0.24	
0	0	0	0	0	0.28	

*I=first experimental block, II=second experimental block.
SOD and CAT were included in the enzyme mixture, GSH, GSH-Px and Plase A2 were included in the protoplast culture medium. The concentrations are coded as given in Table 1. Block I and Block II represent 2 different days. PE values were determined from 1 dish.

Table 4. The effect of ASA (20mg/l) on the PE and the regeneration frequency of protoplasts from a 14 wk old suspension line Lp22KK of *Lolium perenne* L.

		number of shoots/3.5x105 protoplasts (±SE)	
ASA	PE	green	albino
+	0.06 (3)*	8 (1.2)	19 (3.8)
-	0.03 (3)	21 (2.1)	12 (5.5)

* Number of dishes.

Table 5. A set of optimal antioxidant concentrations for the PE of suspension-derived perennial ryegrass protoplasts as identified by the fitted response surface.

		sets of calculated optimal conditions				
antioxidants				**origin**		
SOD	(u/ml)	50	100	**150**	200	250
CAT	(u/ml)	600	800	**1000**	1200	1400
GSH	(mM)	1.0	1.0	**1.0**	1.0	1.0
GSH-PX	(u/ml)	5.0	5.0	**5.0**	5.0	5.0
Plase A2	(u/ml)	85	80	**75**	70	65

The ratio of SOD;CAT;GSH;GSH-PX;Plase A2 is required to be -2;-1;0;0;1. For step width see Table 1.

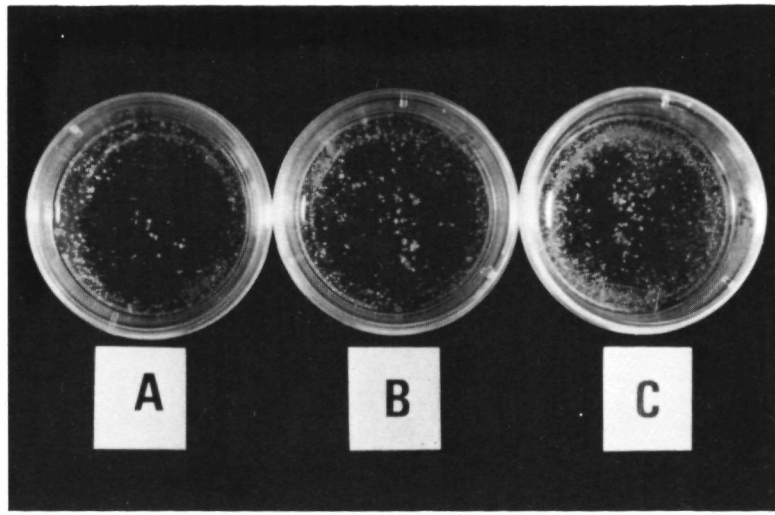

Figure 4. The effect of antioxidants on the PE of suspension-derived protoplasts of *Lolium perenne* L. (line 9A). Microcalli from protoplasts after 3 weeks of culture: [A] isolated and cultured without antioxidants, [B] isolated in the presence of SOD and CAT, [C] isolated in the presence of SOD and CAT and cultured in the presence of GSH, GSH-Px and Plase A2.

DISCUSSION

The conditioning of the protoplast culture medium appeared to be crucial for the proliferation of regeneration-competent calli from protoplasts of young suspension cultures. Furthermore, an important improvement of the PE was achieved by the addition of antioxidants, involved in the reduction of peroxides, to the protoplast isolation and the conditioned culture medium. The use of an experimental design which allows testing of 5 antioxidants simultaneously, appeared to be an essential tool for the determination of optimal concentrations and interactions between antioxidants.

Conditioned medium

In perennial ryegrass the addition of conditioned medium to the protoplast culture medium increased the PE of protoplasts from older suspension cultures and was indispensable for the formation of microcalli from protoplasts of young morphogenic suspension cultures. Although the addition of freshly prepared suspension medium occasionally improved the PE, the major and reproducible effect on the PE was caused by the presence of conditioning factors. These results support those that have been reported earlier for maize (Somers et al., 1987) and *Dactylis* (Horn et al., 1988) and indicate that conditioning factors are

released into the medium by fast-growing suspension culture cells. The characterization of the chemical nature of the conditioning factor (CF) in the maize suspension culture showed that the CF has a non-peptide character and might be an oligosaccharide (Birnberg et al., 1988). However, whether CF from perennial ryegrass has the same chemical nature as CF's in maize cannot be concluded and needs further investigation.

O-acetylsalicylic acid

The addition of ASA to the protoplast culture medium improved the colony formation frequency of perennial ryegrass protoplasts, but not the regeneration frequency. Compared with maize (Carswell, 1989), the effect of ASA on the PE of perennial ryegrass protoplasts was less pronounced but was exerted at 5-10 times lower concentrations. It has been shown that ASA suppressed the formation of ethylene during protoplasts isolation from potato shoots (Perl et al. 1988) and ACC-induced ethylene release from pear cells (Leslie and Romani, 1986). Our results do not permit any conclusion on the mode of action of ASA within the *Lolium* protoplasts system.

Vitamins C and E

At higher concentrations of vitamin C the protoplast yield increased while the PE decreased. In plant cells, vitamin C is an important antioxidant that inhibits lipid peroxidation, either alone or in combination with other antioxidants (Larson, 1988). Simultaneously, vitamin C might stimulate lipid peroxidation by reducing Cu^{2+} or Fe^{3+} ions, making reduced metal ions available to catalyze the conversion of peroxides into reactive hydroxyl or peroxy radicals (Halliwell and Gutteridge, 1989). However, the latter process has been described to occur only at low concentrations of vitamin C, while at high concentrations the scavenging effect dominates over the pro-oxidative action of vitamin C. In this respect the observed negative effect on the PE at higher concentrations of vitamin C is difficult to explain.

The addition of vitamin E to the protoplast isolation or culture medium did not affect the PE of perennial ryegrass protoplasts. In biological membranes, e.g. of chloroplasts, vitamin E functions as a chain breaking antioxidant by reducing intermediate lipid peroxy and alkoxy radicals. The sodium salt of vitamin E, as used in this experimental system, is a polar molecule and may not have reached effective concentrations in the non-polar lipid bilayer in the protoplast membrane.

A synergism between vitamins E and C in trapping free radicals has been suggested to be active in several plant systems (Finck and Kunert, 1985; Kunert and Ederer, 1985). Under the described experimental conditions we could not find any indication for a synergism between vitamin E and C.

n-PG

When cells are injured, lipid peroxides can be produced by lipoxygenases. n-PG exerts its antioxidant effect through the inhibition of lipoxygenase and its ability to bind iron (Halliwell, 1989). However, within this *Lolium* protoplast system the antioxidant properties of n-PG could not be detected.

SOD, CAT, GSH, GSH-Px and Plase A2

Investigation of the combined action of antioxidants in a response surface design led to the identification of a set of optimal concentrations as opposed to just one. A budgetary application of this result is, that by changing the concentrations in a certain direction, the most expensive antioxidants (e.g. SOD) can be added at the most economical concentrations, while still maintaining an optimal PE. In addition, the patterns of interaction between the antioxidants, as reflected by the predicted effects on the PE when the concentrations of certain antioxidants are changed, allow a discussion on the mode of action of the antioxidants within the *Lolium* protoplast system.

The ratio SOD;CAT;GSH;GSH-Px;Plase A2=2;1;0;0;-1 reflects a situation where the PE of protoplasts remains optimal when the concentrations of SOD and CAT are increased or decreased in a reverse direction as the concentrations of Plase A2. These results indicate that the combined effects of SOD and CAT are compensatory to the effect of Plase A2. In plant cells the enzyme catalase converts hydrogen peroxide (H_2O_2) to H_2O. H_2O_2 is not considered a very reactive molecule, but in the presence of traces of reduced metal ions H_2O_2 is converted via the so-called Fenton reaction to the reactive hydroxyl radical OH$^{\cdot}$ (Halliwell and Gutteridge, 1989). Hydroxyl radicals are strong enough to initiate the process of lipid peroxidation. Superoxide dismutase is an enzyme that is ubiquitous in aerobic organisms and protects cells from oxidative damage caused by the superoxide radical O_2^-. SOD catalyzes the conversion of O_2^- to H_2O_2. The main action of O_2^- is the reduction of free and protein-bound metal ions, making these available and active to catalyze the above mentioned Fenton reaction (Halliwell and Gutteridge, 1989). Thus, the formation of hydroxyl radicals is inhibited by both SOD and CAT (Elstner, 1982). In case lipid peroxidation has been initiated and is proceeding, termination of the process might be achieved by the reduction of intermediately formed lipid peroxides. In a reaction that uses GSH as a substrate, such lipid peroxides can be reduced by GSH-PX. This peroxidation step has been found to function in mammalian cells, and the enzyme GSH-Px has not been detected in plant cells. It has been shown that only those peroxides that are not bound to the cell membrane can be reduced by GSH-Px (Halliwell and Gutteridge, 1989). The enzyme Plase A2 cleaves out peroxidized fatty acids, making them available for reduction (van Kuijk, 1987). Our results suggest, that within this *Lolium* protoplast system, the preventative effects of SOD and CAT on lipid peroxidation can be , partly, compensated by the combined

effects of GSH, GSH-Px and Plase A2. For termination and repair of lipid peroxidation the concentration of Plase A2 appears to be crucial.

Our results support those obtained by Ishii (1987), who demonstrated that during enzymatic isolation of protoplasts from oat leaves $O2^-$ was formed and H_2O_2 accumulated in the enzyme mixture. Furthermore, it was shown that the addition of SOD and CAT to the protoplast isolation medium of suspension-cultured rice cells improved the PE of cultured protoplasts, and that the PE could be further improved by the addition of GSH, GSH-PX and Plase A2 to the protoplast culture medium (Ishii, 1988).
The statistical approach as described in this study for determining the effects of antioxidants is recommended to be used when many variables have to be tested, e.g. when optimizing tissue culture procedures. Instead of testing one variable after the other, the experimental design allows testing of all factors simultaneously, giving information on optimal concentrations of each factor and the occurrence of possible interactions between factors.

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REFERENCES

Birnberg PR, Somers DA, Brenner ML (1988) Characterization of conditioning factors that increase colony formation from "Black Mexican Sweet Corn" protoplasts. J Plant Physiol 132:316-321

Carswell GK, Johnson CM, Shillito RD, Harms CT (1989) O-acetylsalicylic acid promotes colony formation from protoplasts of an elite maize inbred. Plant Cell Reports 8:282-284

Cochran WG, Cox GM (1957) Experimental designs. 2nd ed. John Wiley & Sons, New York, Chichester, Brisbane, Toronto, Singapore

Creemers-Molenaar J, Van der Valk P, Loeffen JPM, Zaal MACM (1989) Plant regeneration from suspension cultures and protoplasts of *Lolium perenne* L. Plant Sci 63:167-176

Creemers-Molenaar J, Van Oort Y (1990) Antioxidants influence plating efficiency and microcallus growth of protoplasts in *Lolium perenne* L. In: Nijkamp HJJ, van der Plas LHW, van Aartrijk J (Eds.): Progress in plant cellular and molecular biology, Proceedings VIIth International Congress on Plant Tissue and Cell Culture. Kluwer Academic Publishers, Amsterdam. pp 44-49

Cutler AJ, Saleem M, Coffey MA, Loewen MK (1989) Role of oxidative stress in cereal protoplast recalcitrance. Plant Cell Tissue and Organ Culture 18:113-127

Elstner EF (1982) Oxygen activation and oxygen toxicity. Ann Rev Plant Physiol 33:73-96

Finckh BF, Kunert KJ (1985) Vitamins C an E: An antioxidant system against herbicide-induced lipid peroxidation in higher plants. J Agric Food Chem 33:574-577

Frearson EM, Power JB, Cocking EC (1973) The isolation, culture and regeneration of Petunia leaf protoplasts. Dev Biol 33:130-137

Halliwell B, Gutteridge JMC (1989) Free radicals in biology and medicine, Halliwell, B. and J.C.M. Gutteridge (eds.), Clarendon Press, Oxford.

Horn ME, Conger BV, Harms CT (1988) Plant regeneration from protoplasts of embryogenic suspension cultures of orchard grass (*Dactylis glomerata* L.). Plant Cell Reports 7:371-374

Ishii S (1987) Generation of active oxygen species during enzymic isolation of protoplasts from oat leaves. In Vitro Cell Dev Biol 23:653-658

Ishii S (1988) Factors influencing protoplast viability of suspension-cultured rice cells during isolation process. Plant Physiol 88:26-29

Krens FA, Jamar D, Rouwendal GJA, Hall RD (1990) Transfer of cytoplasm from new *Beta* CMS sources to sugar beet by asymmetric fusion. 1. Shoot regeneration from mesophyll protoplasts and characterization of regenerated plants. Theor Appl Genet 79:390-396

van Kuijk FJGM, Sevanian A, Handelman GJ, Dratz EA (1987) A new role for phospholipase A2: protection of membranes from lipid peroxidation damage. TIBS 12:31-34

Kunert KJ, Ederer M (1985) Leaf aging and lipid peroxidation: The role of the antioxidants vitamin C and E. Physiol Plant 65:85-88

Larson RA (1988) The antioxidants of higher plants. Phytochemistry 27 (4):969-978

Leslie CA, Romani RJ (1986) Salicylic acid: A new inhibitor of ethylene biosynthesis. Plant Cell Reports 5:144-146

Perl A, Aviv D, Galun E (1988) Ethylene and in vitro culture of potato: suppression of ethylene generation vastly improves protoplast yield, plating efficiency and transient expression of an alien gene. Plant Cell Reports 7:403-406

Saleem M, Cutler AJ (1987) Stabilizing corn leaf protoplasts with n-propyl gallate. J Plant Physiol 128:479-484

Schröder R, Gartner F, Steinbrenner B, Knoop B, Beiderbeck R (1989) Viability factors in plant suspension cultures-some properties. J Plant Physiol 135:422-427

Shillito RD, Carswell GK, Johnson CM, Dimaio JJ, Harms CT (1989) Regeneration of fertile plants from protoplasts of elite inbred maize. Bio/Technology 7:581-587

Somers DA, Birnberg PR, Petersen WL, Brenner ML (1987) The effect of conditioned medium on colony formation from "Black Mexican

Sweet" corn protoplasts. Plant Sci 53:249-256
Yamada Y, Zhi-Qi Y, Ding-Tai T (1986) Plant regeneration from protoplast-derived callus of rice (*Oryza sativa* L.). Plant Cell Reports 5:85-88

CHAPTER 6

ASYMMETRIC PROTOPLAST FUSION AIMED AT INTRASPECIFIC TRANSFER OF CYTOPLASMIC MALE STERILITY (CMS) IN *LOLIUM PERENNE* L.

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Molecular & General Genetics (submitted)

SUMMARY

Techniques have been developed for the production of cybrids in *Lolium perenne* (perennial ryegrass). Gamma-irradiated protoplasts of a cytoplasmically male sterile breeding line of perennial ryegrass (B200) were fused with iodoacetamide-treated protoplasts of a fertile breeding line (Jon 401). After fusion twenty five putative cybrid calli were characterized to determine the mitochondrion type and the composition of the nuclear genome. Analysis of PGI isozyme profiles and determination of the ploidy level by flow cytometry indicated that all calli tested essentially contained the nuclear DNA of the fertile line. However, the presence of parts of the nuclear DNA from the sterile line could not be excluded. Southern blotting of total DNA isolated from the parental lines and putative cybrids, combined with hybridizations using the mitochondrial probes *cox*1 and *atp*6, revealed that the mitochondria of the calli originated from the fertile line (5 calli), or from the sterile line (5 calli) or from both parental lines (15 calli). The hybridization patterns of the mtDNA from the cybrid calli showed extensive quantitative and qualitative variation, suggesting that fusion-induced inter- or intramolecular mitochondrial recombination had taken place.

INTRODUCTION

For genetic improvement of a given cultivar, agronomically important traits present in other cultivars or related species can be incorporated by sexual crossing. However, if sexual crossing barriers exist between plants, the transfer of desirable genes from wild relatives to cultivated plants by conventional breeding is prevented. By protoplast fusion the nuclear and cytoplasmic genomes of plants from different genetic origins can be rapidly combined in one step. Somatic hybrid plants have been regenerated from fusions between different species, genera and tribes (Glimelius et al., 1991). Depending on the taxonomic distance, such hybrid plants often show reduced fertility and seed set. However, combined with suitable selection pressure such plants might serve as bridges for the tranfer of specific traits to crop plants (Glimelius, 1991).

Several agronomically important traits, e.g. cytoplasmic male sterility (CMS), herbicide resistance, nectar production and resistance to fungal toxins, have been shown to be encoded by organellar DNA (Kumar and Cocking, 1987). In the majority of higher plants, cytoplasmic organelles are maternally inherited and thus, introduction of such cytoplasmically-encoded traits into a cultivar via breeding requires many backcrosses. By asymmetric protoplast fusion the cytoplasms of two cells can be combined with the nucleus of only one of the parental lines. After regeneration, plants with the desired nucleus/cytoplasm combination can be identified. This technique requires that prior to fusion the nucleus of the cytoplasm donor line is eliminated, either by high-speed

centrifugation or by irradiation (gamma- or X-rays) and, that the recipient protoplasts are inactivated by e.g. iodoacetate or iodoacetamide. Thus, after fusion, the unfused or autofused enucleate and inactivated protoplasts die, whereas, by a process of metabolic complementation, viable cybrids can be obtained and can divide in culture (Sidorov et al., 1981). Cybrid plants have been obtained in several dicotyledonous species such as *Nicotiana* and *Petunia* and cybrid plants with improved breeding value have been produced in *Brassica* and *Solanum* species (Kumar and Cocking, 1987).

Recent progress in the culture and regeneration of protoplasts from monocotyledonous species has stimulated research to apply protoplast fusion techniques in cereals and grasses. Somatic hybridization of *Oryza* has been attempted with wild *Oryza* species (Finch et al., 1990, Hayashi et al., 1988), with barnyard grass (Terada et al., 1987) and between anther-derived haploid cultivars of *O. sativa* (Toriyama and Hinata, 1988). Fertile hybrid plants have been obtained from fusions of *O. sativa* (+) *O. eichingeri*, *O. sativa* (+) *O. officinalis* (Hayashi et al., 1988) and haploid (+) haploid fusions within *O. sativa* (Toriyama and Hinata, 1988). Somatic hybrid callus lines have been recovered from fusions of *Panicum americanum* (+) *Panicum maximum* (Ozias-Akins et al., 1986), *Panicum americanum* (+) *Saccharum officinarum* (Tabaeizadeh et al., 1986) and *Triticum monococcum* (+) *Pennisetum americanum* (Vasil et al., 1988). With the aim of transferring CMS within *O. sativa*, cybrid plants have been obtained by asymmetric somatic hybridization (Yang et al., 1988, 1989, Akagi et al., 1989, Kyozuka et al., 1989).

In perennial ryegrass (*Lolium perenne* L.) a stable CMS-type which originated from a sexual crossing of *L. perenne* x *L. multiflorum* is available (Wit, 1974) and has been used in a breeding program aimed at the production of F_1-hybrid seed. Transfer of this CMS-type to other valuable breeding lines by asymmetric protoplast fusion might greatly enhance the perspectives of this breeding program. Recent progress in the regeneration of plants from protoplasts of *L. perenne* (Creemers-Molenaar et al., 1989, Creemers-Molenaar et al., 1991) allow the application of protoplast fusion techniques in this species. In this study we investigated the possibility of transferring cytoplasmic male sterility from a sterile to a fertile perennial ryegrass breeding line by donor/recipient protoplast fusion.

MATERIALS AND METHODS

Plant material

The cytoplasm donor line used in this study was the CMS inbred line B200 and the recipient line was a selected, fully-fertile breeding line Jon 401. Both lines were provided by Barenbrug Research, Wolfheze, The Netherlands. Suspension cultures were initiated either from immature inflorescence-

derived callus (B200) or directly from mature embryos (Jon 401), as described previously (Creemers-Molenaar et al., 1989). A 3 year old suspension culture Lp9A (derived from B200), and a 10 month old suspension culture Lp25 (derived from Jon 401), were used in fusion experiments. At the time of experimentation, both cultures had lost the potential for plant regeneration.

Protoplast isolation and treatments

Protoplasts were isolated by overnight incubation in CPW-enzyme solution (Creemers-Molenaar et al., 1989) and cultured in conditioned RY-2 medium (Creemers-Molenaar et al., submitted). Prior to fusion, protoplasts from suspension culture Lp9A were washed twice, resuspended in CPW13M medium and then gamma-irradiated at doses in a range of 0-30 krad. The intensity of the ^{60}Co radiation was 150 krad/h. During transport and irradiation the protoplasts were kept on ice. After irradiation the protoplasts were centrifuged and resuspended in CPW13M-medium.

Protoplasts from suspension Lp25 were treated with different concentrations of iodoacetate or iodoacetamide. The protoplasts were washed only once after overnight enzyme-incubation and resuspended in CPW13M medium at a density of $5x10^5$/ml. Freshly prepared stock solutions (0.1 mM) of iodoacetate or iodoacetamide in CPW13M medium were added to the protoplast suspensions to give the final concentrations as described in the Results section. After incubation for 10 min at 4°C in the dark, the protoplasts were centrifuged for 5 min, washed 3 times and, finally, resuspended in CPW13M medium.

PEG-mediated protoplast fusion

Protoplast fusion was performed using polyethylene glycol (PEG) as the fusing agent, according to the method of Gilmour et al. (1989) with minor modifications. Mass fusions were carried out in 10 ml polystyrene tubes (Greiner) using 1.0 x 10^6 protoplasts from each parent in a final volume of 0.3 ml CPW13M medium. The PEG solution (0.4 ml, 30%, PEG6000 from Serva) was added dropwise, without mixing, and the tubes were incubated undisturbed for 30 min at room temperature. Aggregated protoplasts were induced to fuse by the addition of 0.8 ml high pH/Ca^{2+} solution (Keller and Melchers, 1973). After incubation for 10 min the protoplasts were washed 3x in CPWC13M medium (CPW13M supplemented with 0.74% $CaCl_2$ $2H_2O$) and finally cultured in conditioned RY-2 medium. Control treatments consisted of : 1) Separate culture of gamma-irradiated and iodoacetamide/iodoacetate treated protoplasts 2) Separate culture of autofused, treated protoplasts and 3) Cocultivation of autofused, treated protoplasts.

Southern blotting and hybridization

Total DNA from fusion-derived calli and from protoplast-

derived calli from the parental lines was essentially extracted as described by Mettler (1987). Callus tissue (0.3 g) was homogenized in liquid nitrogen with 400 µl extraction buffer using a pestle and mortar. The aqueous phase of the phenol extraction was treated with 0.1 volume (96% v/v) ethanol to reduce contamination with polysaccharides. Precipitated DNA was redissolved in 60 µl TE (10 mM Tris, 1mM EDTA, pH 8.0). DNA was digested with *Eco*R1, using a 2x higher concentration than was recommended by the manufacturer and in the presence of spermidine (final concentration 2.0 mM). Incubation was for 2-3 hrs at 37°C. Southern blotting and DNA hybridizations were performed according to the protocol of Kreike et al. (1990). Heterologous probes, coding for the mitochondrial genes *atp6* (atpase subunit 6, Dewey et al., 1985) and *cox1* (cytochrome c oxidase subunit 1, Isaac et al., 1985), were labeled with biotin as recommended by the manufacturer (Boehringer Mannheim Co.) and used for hybridization. The light-emitting substrate AMPPD (Tropix, Bedford MD) for the enzyme alkaline phosphatase was used, permitting the detection of DNA-DNA hybrids by luminography (Kreike et al., 1990).

Isozyme analysis

Protoplast-derived calli from the parental lines and calli obtained from fusions between these lines were analysed for isozyme patterns 10 days after the 8th subculture (9 months after the fusion experiment). Samples were prepared by homogenizing 0.3 g callus with 20 µl extraction buffer in microfuge tubes, using a power-driven grinding pestle. The extraction buffer was a 0.1 M Tris-HCl at pH 7.1, supplemented with 3% (w/v) sucrose, 1% (w/v) dithiothreitol (DTT) and 0.04% (w/v) amidoblack. The homogenates were centrifuged for 5 min at 1100 x g after which 1 µl of the supernatant was used for electrophoresis. For electrophoresis, the native-PAGE PhastSystem from Pharmacia was used following the procedure as developed by Van Dreven and Booy (personal communication, CPRO, Wageningen). The gel separation media were PhastGel homogeneous 7.5 medium and PhastGel native buffer strips. The gels were run for 60 min and stained for phosphoglucoisomerase (PGI) and acid phosphatase (ACP) directly after the end of the run. For PGI, the gels were incubated for 30 min at 37°C in the dark in 20 ml enzyme-staining solution (Hayward and McAdam, 1977).For ACP, the gels were incubated for 2-3 hrs at room temperature under indirect light conditions in 20 ml enzyme-staining solution (Ostergaard et al., 1985). After staining, the gels were washed with tap water, incubated in 2.5% (v/v) glycerine for 3 min and dried at 37°C.

Ploidy level

The ploidy levels of the calli were determined 7 months after protoplast fusion by flow cytometry. Nuclear suspensions were prepared by chopping 0.25-0.5 g callus with a sharp razor

blade in 2 ml isolation buffer, followed by filtration through a 35 μm mesh nylon filter. The nuclear isolation buffer was prepared as described by Saxena and King (1989) and modified by using 0.2 M sucrose, 10 mM spermine tetrahydrochloride and 0.25 μg/l 4,6-diaminodino-2-phenylindole (DAPI) (Verhoeven, CPRO, Wageningen). A nuclear suspension, prepared from leaves of *Nicotiana plumbaginifolia* plantlets, was used as an internal standard for all the samples. Nuclear suspensions prepared from leaves of greenhouse-grown diploid and tetraploid *L. perenne* varieties were used as controls. The samples were analysed on a Partec PAS-II flow cytometer as described by Verhoeven et al. (1990). The coefficients of variation (CV) were determined as CV(%)=(FWHM/2.355 x MEAN) x 100. In this formula FWHM = full width at half the maximum peak length and, MEAN = peak distance.

RESULTS

Inactivation treatments and PEG fusion

The effects of gamma-irradiation on the plating efficiency of protoplasts from the cytoplasm donor line Lp9A were investigated and the results are shown in Fig. 1. After irradiation of protoplasts at 2.5 krad, the plating efficiency decreased 20x. After irradiation of the protoplasts at 5 krad, microcolonies of 5-10 cells were formed. However, these colonies did not proliferate. No microcolonies were formed after irradiation of the protoplasts at 15 or 30 krad. In further experiments protoplasts of line Lp9A were irradiated at 15 krad prior to fusion.

For inactivation of cells from the recipient line Lp25, freshly isolated protoplasts were initially treated with iodoacetate at 1, 2 and 4 mM. However, after one day of culture only ruptured protoplasts were observed in all treatments and when iodoacetate-treated protoplasts were fused or cocultivated with gamma-irradiated protoplasts from line Lp9A, no microcolonies were formed (results not shown). After treatment of protoplasts from line Lp25 with 1mM iodoacetamide, microcolonies were still formed but at reduced frequency (0.2%) (see Table 1). At higher concentrations, no microcolonies were formed. However, when iodoacetamide-treated protoplasts of line LP25 were subsequently cocultivated with gamma-irradiated protoplasts from line Lp9A, microcolonies were still formed from cells treated with as high as 4 mM iodoacetamide. Nevertheless, further proliferation to calli was only observed when ≤ 2 mM iodoacetamide had been used.

Protoplast fusions were carried out between gamma-irradiated protoplasts from Lp9A and iodoacetamide-treated protoplasts from Lp25 using concentrations in the range 1-7mM

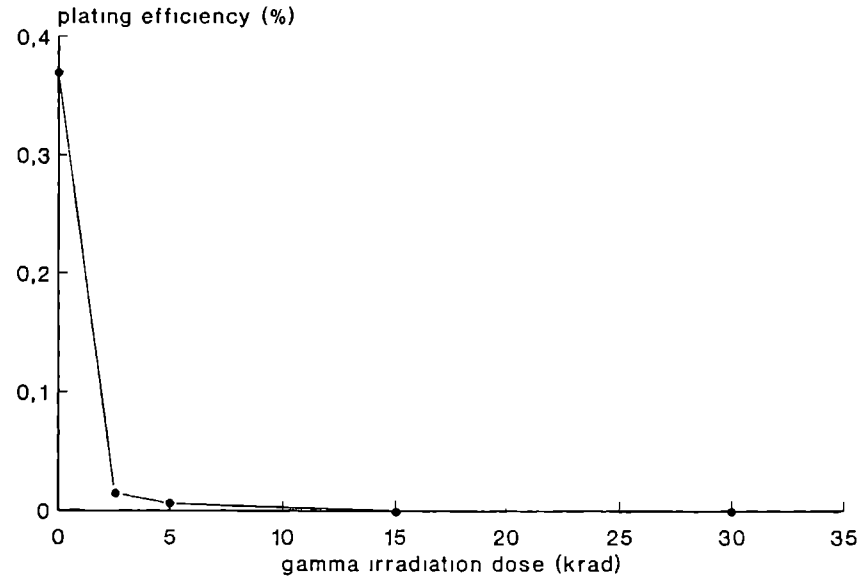

Fig. 1. The effect of gamma-irradiation on the plating efficiencies of protoplasts isolated from suspension cells of *L. perenne* line Lp9A. The intensity of the ^{60}Co irradiation was 150 krad/h.

iodoacetamide. Although no differential staining of the donor and the recipient protoplasts had been applied, due to slow rounding up, microscopic examination immediately after PEG fusion allowed an estimation of the fusion frequency to be made. Irrespective of the iodoacetamide concentration used, the frequency of hetero+homofusion events was 5%. At low concentrations of iodoacetamide (1-2mM), the plating efficiencies and the number of calli that proliferated after fusion, were similar to those obtained in cocultivation (Table 1). However, at higher concentrations (4-7 mM), calli were only formed in the fusion dishes and not in cocultivation dishes. At these higher iodoacetamide concentrations, the plating efficiencies, as well as the number of calli that proliferated after fusion, were low (<0.01% and 2-11 calli/$3.5x10^5$ protoplasts respectively). The results given in Table 1 represent the data from one experiment, this experiment has been repeated twice and in both cases similar results were obtained. A number of the calli that were obtained from one of these experiments, were subcultured for 5-9 months for further characterization.

Table 1. The effect of iodoacetamide-treatment of protoplasts from the recipient line Lp25 on the PE of protoplasts after PEG-mediated fusion with gamma-irradiated protoplasts of the donor line Lp9A in perennial ryegrass.

conc. IA (mM)	culture[2]	cocultivation[3]		fusion[4]	
	pe	pe	calli[1]	pe	calli
0	0.5	/	/	/	/
1	0.2	0.07	9	0.1	11
2	0	0.05	7	0.04	8
4	0	0.02	0	0.01	2
5	0	0	0	<0.01	9
6	0	0	0	<0.01	7
7	0	0	0	<0.01	11

The plating efficiency (pe) was expressed as the percentage of protoplasts that formed microcolonies after 4 weeks of culture and was determined from 2-4 replicate plates.

[1] The number of microcolonies/3.5×10^5 protoplasts which proliferated further to form calli.

[2] Protoplasts of Lp25, cultured after iodoacetamide-treatment.

[3] iodoacetamide-treated protoplasts of Lp25 and gamma-irradiated protoplasts of Lp9A, mixed in a ratio of 1:1 after autofusion.

[4] Fusion of iodoacetamide-treated protoplasts of Lp25 with gamma-irradiated protoplasts of Lp9A.

/ not applicable

IA= iodoacetamide

Characterization of fusion-derived calli

To determine the mtDNA composition of fusion-derived calli, Southern blots were hybridised with *cox*1 and *atp*6 probes. DNA from 25 fusion-derived callus lines and the parental callus lines were examined. The hybridization patterns of 5 fusion-derived calli and calli from the parental lines are shown in Fig. 2. The *cox*1 probe hybridized strongly to fragments of 7.1, 5.6, 4.6, 4.4, 2.4, and 1.3 kb, and weakly to a 3.7-kb and a 3.5-kb fragment, all present in the donor line Lp9A (Fig. 2a). The same probe hybridized strongly to only one fragment of 3.4 kb present in the recipient line Lp25. In callus line 5-1, hybridization with the probe *cox*1 showed 4 fragments (5.6, 4.6, 3.7 and 1.3 kb) corresponding to those of Lp9A and 1 new fragment of 2.7 kb. However, the relative intensity of the 3.7-kb fragment in callus line 5-1 was substantially increased, compared with that of Lp9A. In callus line 5-8 the 7.1-kb and the 2.4-kb fragments of Lp9A were also present, while, compared with callus line 5-1, the stoichiometry of several other signals was clearly different. The hybridization patterns of callus line 5-7 and the recipient line Lp25 were identical, except for a faint extra band at the 4.6-kb fragment position. For callus line 5-3 the probe *cox*1 hybridized to all the fragments mentioned earlier and, in addition, a new hybridizing fragment of 6.2 kb could be identified. The hybridization pattern of callus line 5-9 showed fragments corresponding to the Lp9A-specific 5.6-kb fragment, the amplified 3.7-kb and the novel 2.7-kb fragments as well as a faint signal at the position of the Lp25-specific 3.4-kb fragment. The hybridization patterns of these 5 fusion-derived calli with *cox*1, as shown in Fig. 2a, represent but a small selection from a broad spectrum of qualitative and quantitative variation in mtDNA that was observed among the 25 calli examined. In total, 16 different hybridization patterns could be discriminated when *cox*1 was used as a probe. The amplified 3.7-kb and the novel 2.7-kb fragments were observed respectively in 17 and 16 of the 25 calli that have been examined. The novel 6.2-kb fragment was detected in the DNA from 3 calli. While 5 calli which showed mtDNA hybridization patterns identical to Lp25, none of the calli showed banding patterns identical to Lp9A or to a summation of both parents (Table 2). Treatment of Lp9A with PEG (autofusion) did not alter the DNA hybridization pattern of the calli subsequently obtained (results not shown).

Fig. 2b shows the hybridization patterns of the same calli when *atp*6 was used as the probe. For Lp9A this probe hybridized strongly to fragments of 2.8 kb and 1.5 kb and weakly to a 5.4-kb fragment. For Lp25, *atp*6 hybridized to only a single 2.15-kb fragment. The hybridization pattern of callus line 5-7 was identical to Lp25. DNA from calli 5-1, 5-8 and 5-3 all showed hybridization bands corresponding to the Lp9A-specific fragments of 2.8 kb and 1.5 kb, while this probe only hybridized weakly to the 2.8-kb fragment from DNA of callus

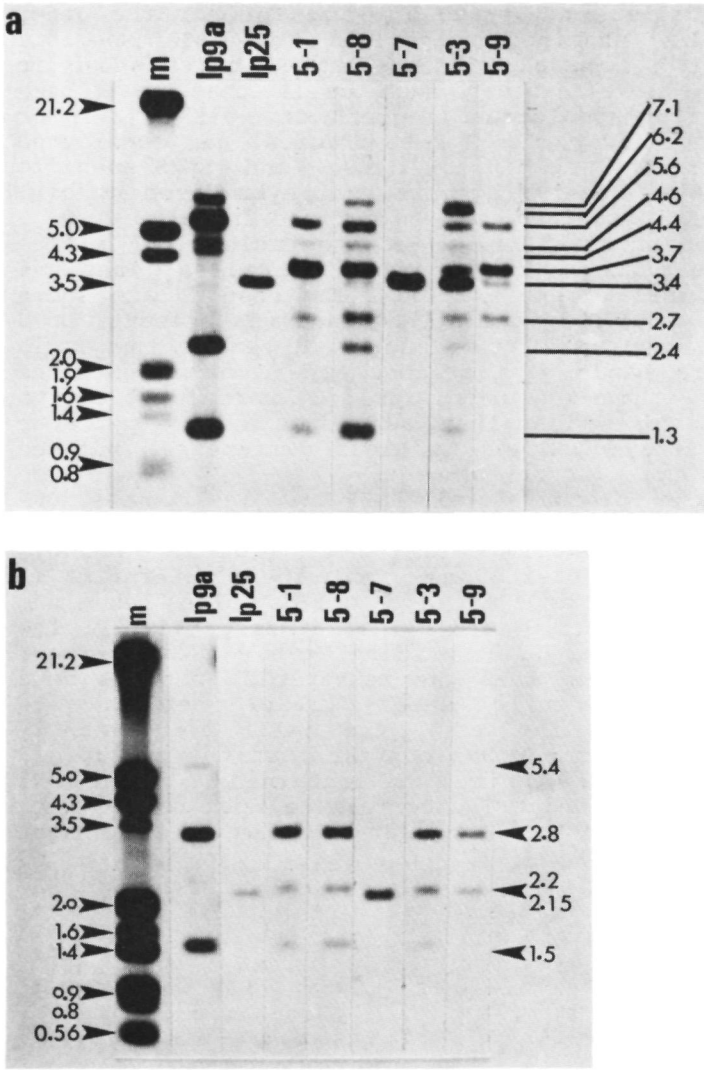

Fig. 2. Southern blot hybridization of *Eco*R1-digested total DNA of protoplast-derived calli from *L. perenne* with the mitochondrial probes *cox*1 (a) and *atp*6 (b). A non-radioactive labeling method was used for the probes and DNA-DNA hybrids were visualized by luminography. M= *Eco*R1+*Hin*d111 digested lambda DNA size markers, Lp9A= CMS parental line, Lp25 = fertile recipient line, 5-1,5-8,5-7,5-3 and 5-9 = fusion-derived calli.

5-9. Neither the calli represented in Fig. 2b, nor the other fusion-derived calli examined, contained the Lp9A specific 5.4-kb fragment. DNA from all fusion-derived calli shown in Fig. 2b, as well as all other such calli that have been examined, had an *atp6*-homologous fragment of either 2.15 or 2.2 kb (results not shown). The 2.2-kb fragment was considered a novel fragment. The number of Lp9A- and Lp25-specific fragments, as well as novel fragments that hybridized to *cox1* and *atp6* of the calli examined, are summarized in Table 2.

To determine the parental origin of the nuclear DNA in the calli, GPI isozyme patterns of the parental callus lines and the fusion-derived calli were examined. The results are shown in Fig. 3. In line Lp9A only one strong band was present. Lp25 showed the same band but at a lower intensity and, in addition, two extra bands were present. The isozyme patterns of all the 25 calli that have been examined, were identical to Lp25. In addition, ACP was analysed, but the isozyme patterns obtained for this enzyme showed no clear differences between the parental lines (results not shown).

To determine the ploidy levels of the calli, the DNA content of isolated nuclei was determined by flow cytometry. The results are summarized in column 2 of Table 2. The CV-values of the DNA histograms of the internal *N. plumbaginifolia* standard and all the callus lines examined varied in a range of 8.8-14.6% and 6.2-14.8% respectively. The ploidy of the callus from the donor line Lp9A was diploid and that of the recipient line Lp25 was tetraploid. Whereas none of the fusion-derived calli were diploid, eight were tetraploid (2n=3.8-4.2x). Twelve of the calli predominantly contained nuclei with a higher DNA content than the tetraploid recipient line Lp25, and one callus contained a lower DNA content. Four calli were chimeric, containing both nuclei with a high DNA content (2n=5.1-6.9x) and a low DNA content (2n=2.8-4.4).

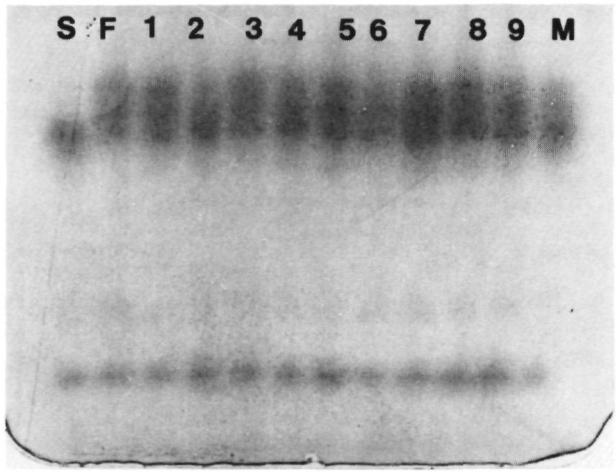

Fig. 3. Zymogram of phosphoglucoisomerase isozyme patterns of protoplast-derived calli from *L. perenne*; CMS parental line Lp9A (S), fertile parental line Lp25 (F), mix of both parental lines (M) and fusion-derived calli 5-7, 5-8, 5-9, 6-1, 6-2, 6-3, 6-4, 6-5, 6-6 (1-9).

Table 2. Summary of the characterization of fusion-derived calli giving proposed parental origin of the nucleus and of the mitochondria.

callus[1] line	ploidy (2n=..x)	mtDNA[2] *cox*1 S	F	R	*atp*6 S	F	R	parental origin nucleus[3]	mitochondria
Lp9A	2	7	0	0	3	0	0	S	S
Lp25	4	0	1	0	0	1	0	F	F
5-1	4	4	0	1	2	0	1	F	S
5-2	3.4	0	1	0	1	0	1	F	SF
5-3	4.1	7	1	2	2	0	1	F	SF
5-4	4.9	0	1	0	0	1	0	F	F
5-5	2.8+5.7	0	1	0	0	1	0	F	F
5-6	6.5	0	1	0	0	1	0	F	F
5-7	2.9+5.1	0	1	0	0	1	0	F	F
5-8	3.1+5.1	6	0	1	2	0	1	F	S
5-9	4.3	2	1	1	1	0	1	F	SF
6-1	4.6	1	1	0	1	1	0	F	SF
6-2	3.8	6	0	1	2	0	1	F	S
6-3	4.7	4	1	2	3	0	1	F	SF
6-4	4.0	3	1	2	2	1	0	F	SF
6-5	4.9	4	1	1	1	0	1	F	SF
6-6	4.7	7	1	1	2	0	1	F	SF
7-1	4.4	1	1	1	1	0	1	F	SF
7-2	3.6	7	0	1	2	0	1	F	S
7-3	5.1	3	1	0	1	1	0	F	SF
7-4	4.4	4	1	0	1	1	0	F	SF
7-5	4.2	0	1	0	0	1	0	F	F
7-6	7.8	2	1	1	1	1	0	F	SF
7-7	4.2	7	1	1	2	1	0	F	SF
7-8	4.2	6	1	1	1	1	0	F	SF
7-9	4.4+6.9	3	1	1	2	0	1	F	SF
7-10	4.0	7	0	1	2	0	1	F	S

[1] Lp9A= sterile donor line, Lp25=fertile recipient line, lines 5-(1-9), 6-(1-6) and 7-(1-10) are fusion-derived calli, iodoacetamide treatments of the recipient line were 5, 6, and 7 mM respectively.

[2] *cox*1: S= 1-7 hybridizing fragments of the sterile line, F= hybridization to the unique Lp25 fragment, R: novel hybridizing fragments of 6.2 and/or 2.7 kb. *atp*6: S= hybridization to 1-3 fragments of the sterile line, F: hybridization to the 2.15 kb fragment of Lp25, R: novel hybridizing fragment of 2.2 kb.

[3] Based on ploidy and the presence of Lp25-specific GPI isozyme bands in all the fusion-derived calli.

DISCUSSION

In this paper a method has been described to achieve asymmetric protoplast fusion in perennial ryegrass, incorporating gamma-irradiation of the donor protoplasts and iodoacetamide inactivation of the recipient protoplasts. Isozyme patterns and ploidy levels of the calli indicated that all 25 fusion-derived calli examined contained the nuclear genome of the fertile recipient line Lp25. Based on the mtDNA analysis, 15 calli contained a mixture of mitochondrial DNA sequences from both lines, 5 calli contained only mitochondrial DNA sequences of the sterile donor line and 5 calli contained only mitochondrial DNA sequences of the fertile recipient line. In addition, the hybridization patterns of Southern blots using *cox*1 and *atp*6 as a probe, revealed that distinct mtDNA rearrangements had taken place in the majority of the calli which contained DNA fragments complementary to the sterile line.

Although the nuclear DNA of all the fusion-derived calli originated from the fertile recipient line, as established through PGI isozyme analysis, it cannot be excluded, considering the, on average, high ploidy levels, that fractions of the nuclear genome of the sterile line were also present. Sidorov et al. (1981) found that after PEG-fusion of gamma-irradiated protoplasts of *N. tabacum* with iodoacetate-treated protoplasts of *N. plumbaginifolia*, 60% of the regenerated plants contained some nuclear genetic material from the irradiated cells. The variable ploidy levels and the chimeric nature of several of the fusion-derived calli, as observed in this study, may have been due to the presence of chromosome fragments of the donor line which induce amplification and/or chromosome loss in the recipient genome. Recently, an efficient procedure for the isolation of cytoplasts from suspension-derived protoplasts of *Lolium perenne* has been developed (Van Ark et al., submitted). Fusion of the recipient protoplasts with cytoplasts, instead of irradiated protoplasts, may be a means to circumvent partial transfer of nuclear DNA from the donor line.

The inactivation treatment of the recipient line was a critical factor. Perennial ryegrass protoplasts showed an extreme sensitivity to iodoacetate and, therefore, this metabolic inhibitor was not suited for inactivation purposes. These results contrast with those obtained for *Nicotiana* (Sidorov et al., 1981), *Solanum* (Kemble et al,. 1986), *Panicum maximum* (Ozias-Akins, 1986) and *Saccharum officinarum* (Tabazaeideh et al., 1986), for which iodoacetate has been applied successfully for the inactivation of protoplasts to obtain cybrids. On the other hand, in perennial ryegrass iodoacetamide prevented growth of the recipient protoplasts, but allowed the proliferation of heterokaryons after fusion with irradiated protoplasts. High concentrations of iodoacetamide (5-7mM) were essential to prevent division of

autofused, iodoacetamide-treated protoplasts in the presence of autofused, irradiated protoplasts. The irradiated protoplasts appeared to exert a feeder effect on the iodoacetamide-treated protoplasts.

The hybridization patterns of cybrid *Lolium* calli with *cox*1 showed particular qualitative, and a range of quantatative changes, as compared to calli of the parental lines. The DNA hybridization patterns obtained after autofusion were identical to those of protoplast-derived calli. Hence, the mtDNA rearrangements observed in the cybrids resulted from protoplast heterofusion and were not induced by tissue culture itself. This is in contrast with tissue culture-induced mtDNA rearrangements, independent of a fusion process, which have previously been observed in e.g. *Solanum* (Kemble and Shepard, 1984), *Triticum* (Rode, 1987) *Beta* (Brears, 1989) and *Oryza* (Chowdhury et al., 1990).

The extent of variation between the mtDNA hybridization patterns of cybrid calli in perennial ryegrass and the occurrence of novel fragments suggest that intermolecular recombination has taken place (Boeshore et al., 1983). Intermolecular recombination has been demonstrated in somatic hybrids of *Petunia* (Rothenberg et al., 1985). If intermolecular recombination has taken place in *Lolium*, the appearance of the common non-parental 3.7-kb and 2.7-kb fragments in 17 and 16 of the cybrid calli respectively, may indicate the presence of recombination "hot spots". Previously, the occurrence of hot spots of recombination was suggested to explain identical mtDNA restriction patterns in somatic cybrid plants of rice. These patterns differed from the starting material in that several non-parental bands were present (Yang et al., 1988). Alternatively, the heteroplasmic state might have induced preferential amplification of specific mtDNA molecules that were already present at substoichiometric concentrations in the parental lines, as has been demonstrated in hybrids of pearl millet and Guinea grass (Ozias-Akins et al., 1988). However, more extensive mtDNA analysis will be necessary to determine which process caused the observed mtDNA variation in perennial ryegrass cybrid calli.

This is the first report of experiments aimed to obtain intraspecific transfer of cytoplasmic male sterility by asymmetric protoplast fusion in the economically-important forage crop *Lolium perenne*. Cybrid calli were obtained which contained the recipient nuclear DNA at the expected ploidy level and only the mitochondria of the donor line. The high percentage of cybrid calli that was obtained in this study offers promising perspectives for the regeneration of cybrid plants, when the described procedure is applied to regeneration-competent suspension lines.

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REFERENCES

Akagi H, Sakamoto M, Negishi T, Fujimura T (1989) Construction of rice cybrid plants. Mol Gen Genet 215:501-506

Boeshore ML, Lifshitz I, Hanson MR, Izhar S (1983) Novel composition of mitochondrial genomes in *Petunia* somatic hybrids derived from cytoplasmic male sterile and fertile plants. Mol Gen Genet 190:459-467

Brears T, Curtis GJ, Lonsdale DM (1989) A specific rearrangement of mitochondrial DNA induced by tissue culture. Theor Appl Genet 77:620-624

Chowdhury MKU, Schaeffer GW, Smith RL, Debonte LR, Matthews BF (1990) Mitochondrial DNA variation in long-term tissue cultured rice lines. Theor Appl Genet 80:81-87

Creemers-Molenaar J, Van der Valk P, Loeffen JPM, Zaal MACM (1989) Plant regeneration from suspension cultures and protoplasts of *Lolium perenne* L. Plant Sci 63:167-176

Creemers-Molenaar J (1991) Regeneration from protoplasts of perennial ryegrass; progress and applications. In: den Nijs APM and Elgersma A (eds.), Fodder crops breeding: achievements, novel strategies and biotechnology. Proceedings of the 16th meeting of the Fodder Crops Section of Eucarpia, Wageningen, The Netherlands 16-22 November 1990 pp 123-128

Dewey RE, Levings CS III, Timothy DH (1985) Nucleotide sequences of atpase subunit 6 of maize mitochondria. Plant Physiol 79:914-919

Finch RP, Slamet IH, Cocking EC (1990) Production of heterokaryons by the fusion of mesophyll protoplasts of *Porteresia coarctata* and cell suspension-derived protoplasts of *Oryza sativa*: a new approach to somatic hybridization in rice. J Plant Physiol 136:592-598

Gilmour DM, Davey MR, Cocking EC (1989) Production of somatic hybrid tissues following chemical and electrical fusion of protoplasts from albino cell suspensions of *Medicago sativa* and *M. borealis*. Plant Cell Rep 8:29-32

Glimelius K, Fahlesson J, Landgren M, Sjodin C, Sundberg E

(1991) Gene transfer via somatic hybridization in plants. TibTech 9:24-30

Hayashi Y, Kyozuka J, Shimamoto K (1988) Hybrids of rice (*Oryza sativa* L.) and wild *Oryza* species obtained by cell fusion. Mol Gen Genet 214:6-10

Hayward MD, McAdam NJ (1977) Isozyme polymorphism as a measure of distinctiveness and stability in cultivars of *Lolium perenne*. Z Pflanzenzüchtg 81:228-234

Isaac P, Jones VP, Leaver CJ (1985) The maize cytochrome c oxidase subunit I gene: sequence, expression and rearrangement in cytoplasmic male sterile plants. EMBO J 4:1617-1623

Keller WA, Melchers G (1973) The effect of high pH and calcium on tobacco leaf protoplast fusion. Z Naturforsch 28C:737-741

Kemble RJ, Shepard JF (1984) Cytoplasmic DNA variation in a potato protoclonal population. Theor Appl Genet 69:211-216

Kemble RJ, Barsby TL, Wong RSC, Shepard JF (1986) Mitochondrial DNA rearrangements in somatic hybrids of *Solanum tuberosum* and *Solanum brevidens*. Theor Appl Genet 72:787-793

Kreike CM, de Koning JRA, Krens FA (1990) Non-radioactive detection of single-copy DNA-DNA hybrids. Plant Mol Biol Reporter 8(3):172-179

Kumar A, Cocking EC (1987) Protoplast fusion: a novel approach to organelle genetics in higher plants. Amer J Bot 74(8):1289-1303

Kyozuka J, Kaneda T, Shimamoto K (1989) Production of cytoplasmic male sterile rice (Oryza sativa L.) by cell fusion. Bio/Technology 7:1171-1174

Mettler IJ (1987) A simple and rapid method for minipreparation of DNA from tissue cultured plant cells. Plant Mol Biol Reporter 5(3):346-349

Østergaard H, Nielsen G, Johansen H (1985) Genetic variation in cultivars of diploid ryegrass, *Lolium perenne* and *L. multiflorum*, at five enzyme systems. Theor Appl Genet 69:409-421

Ozias-Akins P, Ferl RJ, Vasil IK (1986) Somatic hybridization in the gramineae: *Pennisetum americanum* (L.) K. Schum. (Pearl millet) + *Panicum maximum* Jacq. (Guinea grass). Mol Gen Genet 203:365-370

Ozias-Akins P, Tabaeizadeh Z, Pring DR, Vasil IK (1988) Preferential amplification of mitochondrial DNA fragments in somatic hybrids of the Gramineae. Curr Genet 13:241-245

Rode A, Hartmann C, Falconet D, Lejeune B, Quetier F, Benslimane A, Henry Y, de Buyser J (1987) Extensive mitochondrial DNA variation in somatic tissue cultures initiated from wheat immature embryos. Curr Genet 12:369-376

Rothenberg M, Boeshore ML, Hanson MR, Izhar S (1985) Intergenomic recombination of mitochondrial genomes in a somatic hybrid plant. Curr Genet 9:615-618

Saxena PK, King J (1989) Isolation of nuclei and their transplantation into plant protoplasts. In: Bajaj YPS (ed.), Biotechnology in agriculture and Forestry 9: Plant protoplasts and genetic engineering II. Springer-Verlag, Berlin, Heidelberg, New York. pp. 328-342

Sidorov VA, Menczel L, Nagy F, Maliga P (1981) Chloroplast transfer in *Nicotiana* based on metabolic complementation between irradiated and iodoacetate treated protoplasts. Planta 152:341-345

Tabaeizadeh Z, Ferl RJ, Vasil IK (1986) Somatic hybridization in the Gramineae: *Saccharum officinarum* L. (sugarcane) + *Pennisetum americanum* (L.) K. Schum. (Pearl millet). Proc Nat Acad Sci USA 83:5616-5619

Terada R, Kyozuka J, Nishibayashi S, Shimamoto K (1987) Plantlet regeneration from somatic hybrids of rice (*Oryza sativa* L.) and barnyard grass (*Echinochloa oryzicola* Vasing). Mol Gen Genet 210:39-43

Toriyama K, Hinata K (1988) Diploid somatic-hybrid plants regenerated from rice cultivars. Theor Appl Genet 76: 665-668

Vasil V, Ferl RJ, Vasil IK (1988) Somatic hybridization in the Gramineae: *Triticum monococcum* L. (Einkorn) + *Pennisetum americanum* (L.) K. Schum. (pearl millet). J Plant Physiol 132:160-163

Verhoeven HA, Sree Ramulu K, Dijkhuis P (1990) A comparison of the effects of various spindle toxins on metaphase arrest and formation of micronuclei in cell-suspension cultures of *Nicotiana plumbaginifolia*. Planta 182:408-414

Wit F (1974) Cytoplasmic male sterility in ryegrasses (*Lolium* spp.) detected after intergeneric hybridization. Euphytica 23:31-38

Yang Z-Q, Shikanai T, Yamada Y (1988) Asymmetric hybridization between cytoplasmic male sterile (CMS) and fertile rice (*Oryza sativa* L.) protoplasts. Theor Appl Genet 76:801-808

Yang Z-Q, Shikanai T, Mori K, Yamada Y (1989) Plant regeneration from cytoplasmic hybrids of rice (*Oryza sativa* L.). Theor Appl Genet 77:305-310

SUMMARY AND CONCLUDING REMARKS

The aim of this research was to develop a method for the intraspecific transfer of CMS by protoplast fusion in *Lolium perenne*. Therefore, a reproducible system for plant regeneration from protoplasts, as well as a method for asymmetric protoplast fusion and characterization of fusion products had to be developed.

During the course of this study plant regeneration from protoplasts has been achieved for 4 out of 30 cultivars/breeding lines tested. Fully fertile, protoplast-derived plants have been obtained from one cultivar. A crucial factor for successful plant regeneration from protoplasts is the availability of finely dispersed, regeneration-competent suspension cultures. The establishment of such suspension cultures is difficult and requires considerable experience. In addition, once an established suspension culture has been obtained, the competence for plant regeneration gradually decreases to zero within 25-30 weeks after suspension culture initiation, with only a few exceptions. It could be shown that the observed reduction of the morphogenic response is correlated with a decrease in the ploidy level of the suspension cultures. However, determination of the ploidy levels of green shoots, regenerated from sub-diploid suspension cultures, indicate that these plantlets originate from normal diploid cells. In contrast, albino shoots obtained from the same cultures are predominantly polyploid, mixoploid or aneuploid. It is concluded that the occurrence of albino shoots from perennial ryegrass suspension cultures is indicative of the progression of chromosomal variation, which ultimately results in the complete loss of the morphogenic capacity of the cells. To extend the regeneration competence period of suspension cultures cold storage appeares to be convenient. With the aim of making the establishment of suspension cultures more reproducible, genotypes have been selected among regenerated plants which show a good response for callus induction and, subsequently, suspension culture initiation. However, irrespective of these improvements, the morphogenic potential of perennial ryegrass suspension cultures remains difficult to control.

Somatic hybridization experiments, using treatments with chemical agents such as polyethylene glycol or with electrofusion, have been shown to reduce the plating efficiency of protoplasts. Therefore, experiments were carried out to optimize the plating efficiency of cultured, untreated protoplasts. It was shown that conditioning of the protoplast culture medium with medium from fast-growing suspension cultures, is indispensable for the formation of microcalli from protoplasts from young morphogenic suspension cultures and, in addition, enhances microcallus formation from protoplasts from older suspension cultures. It is concluded that conditioning factors, released into the medium by fast-

growing suspension cells, exert a feeder effect on the cultured protoplasts. However, the chemical nature of the conditioning factor(s) was not determined and needs further investigation. The addition of O-acetylsalicylic acid, a compound that has been shown to inhibit the formation of ethylene, to the protoplast culture medium improves the formation of non-morphogenic callus from protoplasts. Since we were not able to measure ethylene in the head space of flasks in which protoplasts were cultured, using gas chromatography, the involvement of ethylene in the formation of microcalli from *Lolium* protoplasts could not be established. The addition of antioxidants (superoxide dismutase, catalase, glutatione, glutathione peroxidase and phospholipase A2) involved in the reduction of peroxides to the protoplast isolation medium and the culture medium, in specific concentration ratios, increases the plating efficiency up to five-fold. It is concluded that during protoplast isolation and the initial culture period, oxidative stress occurres that resultes in membrane damage by the process of lipid peroxidation. The addition of antioxidants prevents the initiation and prolongation of the lipid peroxidation and thus facilitates the repair of already damaged membranes and increases the plating efficiency.

Although important progress was made in the culture and regeneration of perennial ryegrass protoplasts by optimizing the protoplast isolation and culture medium, the acquisition of regeneration-competent protoplasts largely depends on the presence of high numbers of morphogenic cells in the suspension culture from which the protoplasts are isolated. In this respect, further research on the characterization and selection of regeneration-competent cells in heterogeneous suspension cultures is warranted.

During the subsequent part of this research project a procedure for asymmetric fusion of perennial ryegrass protoplasts was determined. Perennial ryegrass protoplasts withstand fusion with the chemical agent polyethylene glycol better than electrofusion. Prior to fusion, the nuclear DNA of protoplasts from the sterile donor line was damaged by gamma-irradiation and the cytoplasm of protoplasts from the fertile recipient line was inactivated by iodoacetamide. Fusion-derived calli were analysed by flow cytometry, isozyme patterns and Southern blotting using mitochondrial DNA probes for hybridization. Cybrid calli were obtained which contain the nuclear DNA of the fertile recipient line and the mitochondria of the donor line. However, the hybridization patterns of the mtDNA from these cybrid calli show extensive quantitative and qualitative variation, suggesting that fusion-induced inter- or intramolecular mitochondrial DNA recombination had taken place.

In conclusion, a reproducible system for plant regeneration from protoplasts of *Lolium perenne* has been developed. Asymmetric protoplast fusion experiments yielded cybrid calli containing the nuclear DNA of at least the fertile recipient line and the mtDNA of the male sterile donor line. From these calli, regeneration of plants has not been obtained. Further experiments, in which protoplasts from regeneration-competent suspension lines are used as the fusion partners, should prove the feasibility of using protoplast fusion techniques to transfer cytoplasmic male sterility between perennial ryegrass plants.

SAMENVATTING

Het doel van dit onderzoek was de overdracht van cytoplasmatische mannelijke steriliteit (CMS) via protoplastenfusie binnen *Lolium* species. CMS is een door de mitochondriën gecodeerde eigenschap die fenotypisch tot expressie komt wanneer in het kern-DNA geen herstellergenen aktief zijn. Mannelijke steriliteit is de eigenschap dat geen functioneel pollen wordt gevormd en de aanwezigheid van deze eigenschap in een van de ouderlijnen is essentieel bij de produktie van F_1 hybride zaad.

Overdracht van CMS via protoplastenfusie impliceert dat uit de fusieprodukten weer intakte planten geregenereerd moeten kunnen worden. Een reproduceerbare methode voor de regeneratie van planten uit protoplasten moet dus voorhanden zijn. Bij monocotyle gewassen, o.a. grassen en granen, is regeneratie van protoplasten die direkt uit planteweefsel worden geïsoleerd tot nu toe niet mogelijk gebleken. Alleen als snel groeiende, regeneratie-competente suspensieculturen worden gebruikt voor de isolatie van protoplasten is regeneratie van planten mogelijk.

Hoofdstuk 1 is een inleiding op de toepassing van celbiologische en moleculair biologische technieken bij de veredeling van raaigrassen. De vorderingen op dit gebied worden besproken aan de hand van relevante literatuur op het gebied van monocotylen en in het bijzonder van raaigras.

In hoofdstuk 2 wordt het proces van callus inductie uit explantaten van onvolgroeide bloeiwijzen beschreven. Tevens wordt de regeneratie van planten uit het verkregen callus beschreven. Binnen vier *L. perenne* rassen en twee *L. multiflorum* rassen varieerde de frequentie waarmee de explantaten kompakt en/of embryogeen callus vormden respectievelijk van 31-100% en van 8-100%. Voor alle geteste rassen werden zowel groene als albino scheutjes geregeneerd uit kompakt/embryogeen callus. Het percentage explantaten met callus waaruit albino scheutjes werden geregenereerd nam toe met de 2,4-D concentratie in het medium voor callusinductie en was hoger voor explantaten van in het veld gegroeide planten dan van in de kas gegroeide planten. Het aantal albino scheutjes nam toe naarmate het callus verouderde.

Voor het initiëren van suspensieculturen werd gebruik gemaakt van kompakt, regeneratie-competent callus of van uit zaden geprepareerde embryos welke direkt in vloeibaar medium werden gebracht. De eigenschappen van deze suspensieculturen zijn beschreven in hoofdstuk 3. Tevens is voor een aantal van de verkregen suspensieculturen de isolatie en cultuur van protoplasten beschreven. Fijn verdeelde, regeneratie-competente suspensieculturen werden via beide hierboven genoemde methoden verkregen. Over een periode van 25 weken na initiatie nam het regeneratievermogen van de meeste suspensieculturen geleidelijk af tot nul. De sterkste daling van het regeneratie vermogen trad op vanaf het moment dat de

culturen fijn verdeeld werden (15-20 weken na initiatie). Een uitzondering hierop vormde de suspensiecultuur Lp9a, geïnitieerd met hoog-regeneratief callus van de CMS lijn Lp9. Deze cultuur bleef meer dan twee jaar competent voor regeneratie, zij het met lage frequentie. Uit deze cultuur werden protoplasten geisoleerd die in staat waren tot plant regeneratie. Voor de vorming van microcalli uit protoplasten bleek de aanwending van een complex, met organische zuren en vitamines verrijkt medium belangrijk te zijn. Het aanbrengen van een hoge osmolariteit met glucose (910-920 mOsm) i.p.v. mannitol of sucrose bleek essentieel.

In hoofdstuk 4 worden de resultaten beschreven van onderzoek dat erop was gericht de initiatie en regeneratie van suspensieculturen beter te begrijpen en te kunnen kontroleren. Om te onderzoeken of de afname van het regeneratievermogen van suspensieculturen gecorreleerd is aan veranderingen van het ploïdie nivo, werd het DNA-gehalte en de regeneratiefrequentie van suspensies in de tijd gevolgd. Er was een duidelijke correlatie aanwezig tussen de afname van het regeneratievermogen en een afname van het ploïdie nivo in zes van de negen culturen die werden gevolgd. Opmerkelijk was dat uit suspensieculturen met een sterk verlaagd ploïdie nivo groene scheutjes met een normaal ploïdie nivo werden geregeneerd, terwijl het merendeel van de albino scheutjes polyploïd of mixoploïd was. Verder werd onderzocht of de initiatie van suspensieculturen beter reproduceerbaar gemaakt kon worden. Het bleek mogelijk om uit regeneranten van protoplasten en suspensieculturen genotypen te selecteren met goede eigenschappen voor callusinductie en de initiatie van suspensieculturen. Ook bleek dat koudebewaring van suspensieculturen het proces van verlies van regeneratievermogen vertraagde, hetgeen resulteerde in het langer beschikbaar zijn van de culturen voor protoplastering.

Om de cultuur van protoplasten te verbeteren, en de regeneratie vanuit protoplasten voor meerdere cultivars mogelijk te maken, werd een aantal modificaties van het oorspronkelijke protoplast cultuur medium uitgetest. Dit is beschreven in hoofdstuk 5. Toevoeging van 50% gekonditioneerd medium verkregen uit suspensieculturen was essentieel voor de vorming van regeneratie-competente microcalli uit protoplasten die werden geisoleerd uit jonge suspensieculturen. O-acetylsalicyl zuur bevorderde vooral de doorgroei van niet regeneratie-competente protoplasten. Toevoeging van superoxide dismutase en catalase aan de enzymmix en van fosfolipase A2, glutathion en glutathion peroxidase aan het cultuurmedium verhoogde de efficiëntie waarmee protoplasten microcalli vormden aanzienlijk.

Tenslotte is in hoofdstuk 6 de ontwikkeling van een procedure voor asymmetrische protoplastenfusie beschreven. *Lolium* protoplasten bleken beter bestand tegen chemische fusie d.m.v. polyethyleen glycol dan tegen elektrofusie. Voorafgaand aan fusie werd de kern van protoplasten van de steriele lijn

uitgeschakeld door gamma-bestraling en het cytoplasma van protoplasten van de fertiele lijn werd geïnactiveerd d.m.v. joodacetamide. Van meer dan tweehonderd uit fusieëxperimenten afkomstige calli werden er 25 nader gekarakteriseerd. Op grond van flowcytometrie en isoenzymanalyse werd gekonkludeerd dat alle calli het kern DNA van de fertiele ouderlijn bevatten, maar dat overdracht van stukjes kern-DNA van de steriele ouderlijn niet geheel kon worden uitgesloten. Het mitochondriële DNA werd geanalyseerd d.m.v. Southern blotting en hybridisatie met mitochondriële probes. In vijf calli waren alleen mitochondriën van de steriele ouderlijn aanwezig, in 5 calli alleen die van de fertiele lijn en 15 calli bevatten een mengsel van mitochondriën afkomstig van beide ouderlijnen. De mitochondriële hybridisatiepatronen van de cybride calli (calli met nieuwe kern/cytoplasma kombinaties) vertoonden zowel t.o.v. elkaar als ook t.o.v. de steriele ouderlijn een scala van kwantitatieve en kwalitatieve verschillen. Op grond hiervan werd gekonkludeerd dat als gevolg van de fusie, waarbij twee populaties van mitochondriën gemengd worden, inter- of intramoleculaire mitochondriële recombinatie had plaats gevonden.

Tot besluit kan worden gesteld dat een reproduceerbare methode voor de regeneratie van planten uit protoplasten van engels raaigras is ontwikkeld. Uit asymmetrische protoplastenfusies zijn cybride calli verkregen met het kern-DNA van tenminste de fertiele ouderlijn en het mitochondriële DNA van de steriele ouderlijn. Experimenten waarin protoplasten worden gefuseerd die afkomstig zijn van regeneratie-competente suspensieculturen zullen uitsluitsel kunnen geven over de toepasbaarheid van deze methode om CMS binnen Lolium species over te dragen.

CURRICULUM VITAE

De auteur van dit proefschrift werd geboren op 11 december 1951 te Arnhem. Zij volgde het HAVO aan de Nijmeegse Scholen Gemeenschap en vervolgens een HBO opleiding voor medisch microbiologisch analist in Nijmegen. Gedurende acht jaar werkte zij bij het Radboudziekenhuis te Nijmegen aan uiteenlopende onderwerpen als medische microbiologie, ziekenhuishygiëne en transplantatieserologie. In 1979 begon zij met een universitaire studie biologie aan de Faculteit der Wis- en Natuurwetenschappen van de universiteit te Nijmegen. Het doctoraal examen werd afgelegd in 1985, in de hoofdrichting Planten Fysiologie (afdeling Botanie, KU Nijmegen) en in de nevenrichtingen Biohistorie (RU, Utrecht) en Voedingsmiddelen Microbiologie (LU, Wageningen). Aansluitend heeft zij in dienst van de firma Barenbrug aan het onderzoek gewerkt waarvan de resultaten zijn beschreven in dit proefschrift. Vanaf maart 1991 werkt zij bij het Centrum voor Plantenveredelings en Reproductie Onderzoek (CPRO) te Wageningen aan onderzoek dat is gericht op transformatie en regeneratie van recalcitrante gewassen.

Stellingen

1.
Verhoging van de plating efficiency van protoplasten leidt niet altijd tot verhoging van de regeneratiefrequentie.
(Dit proefschrift)

2.
De afname van het regeneratievermogen van suspensieculturen van *Lolium perenne* is gecorreleerd aan afname van het DNA-gehalte van de suspensiecellen.
(Dit proefschrift)

3.
Bij de overdracht van cytoplasmatische mannelijke steriliteit (CMS) via protoplastenfusie is nog onduidelijk in hoeverre de, als gevolg van fusie optredende, recombinatie van het mitochondriële DNA invloed heeft op het tot expressie komen van CMS in geregenereerde planten.
(Dit proefschrift)

4.
Het gebruik van de term celsuspensiecultuur is misleidend wanneer hiermee in feite een fijn verdeelde calluscultuur in vloeibaar medium wordt bedoeld, een omschrijving waaraan in ieder geval alle tot nu toe beschreven suspensieculturen van monocotyle gewassen voldoen.

5.
Behalve de interpretatie van Okada et al. dat verhoging van de transformatiefrequentie van *Nicotiana* protoplasten tijdens de M-fase van de celcyclus het gevolg is van optimale toegankelijkheid van de celkern voor exogeen DNA, zou het ontbreken van een aktief DNA-repairsysteem tijdens deze fase eveneens overwogen moeten worden.

Okada et al., 1986. Mol. Gen. Genet. 205: 398-403.

6.
De geheimzinnigheid waarmee sommige zaadbedrijven hun, vaak nog precompetitieve, biotechnologisch gerichte onderzoek omhullen, belemmert de wetenschappelijke communicatie van de onderzoekers en zal, in tegenstelling tot wat wordt beoogd, tot achterstand leiden in het betreffende vakgebied.

7.
Het is nog de vraag in hoeverre fusies, zowel op protoplastnivo als op instituutsnivo, tot bloeiend resultaat komen.

8.

Het ontbreken van een literair citaat in een proefschrift impliceert niet dat de schrijver van het proefschrift onbelezen is; Het tegenovergestelde geldt ook niet.

9.

In rotsklimmersjargon kan promoveren het beste als volgt worden omschreven: "Die hohe, fast senkrechte, doch gutgriffige Wand hinauf und weiter durch einen langen Kamin mit Verengungen und kleine Überhängen zu einer Scharte wenige meter vor dem Vorgipfel und über den Gipfelgrat mit einer Einschartung zum höchsten Punkt."

Langes G (1979) SO-Kante, Cima Pradidali, 2754 m. In: Dolomiten-Kletterführer, Marmolata-und Palagruppe, p. 104.

Stellingen behorende bij het proefschrift: "Protoplast fusion for intraspecific transfer of cytoplasmic male sterility in perennial ryegrass" door Jantina Creemers-Molenaar, Doorn, 4 november 1991.